Are Structural Analogues to Bisphenol A Safe Alternatives? Comparative In-vitro and In-vivo Approaches to Study Reproductive Toxicity in Male Rats

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIRMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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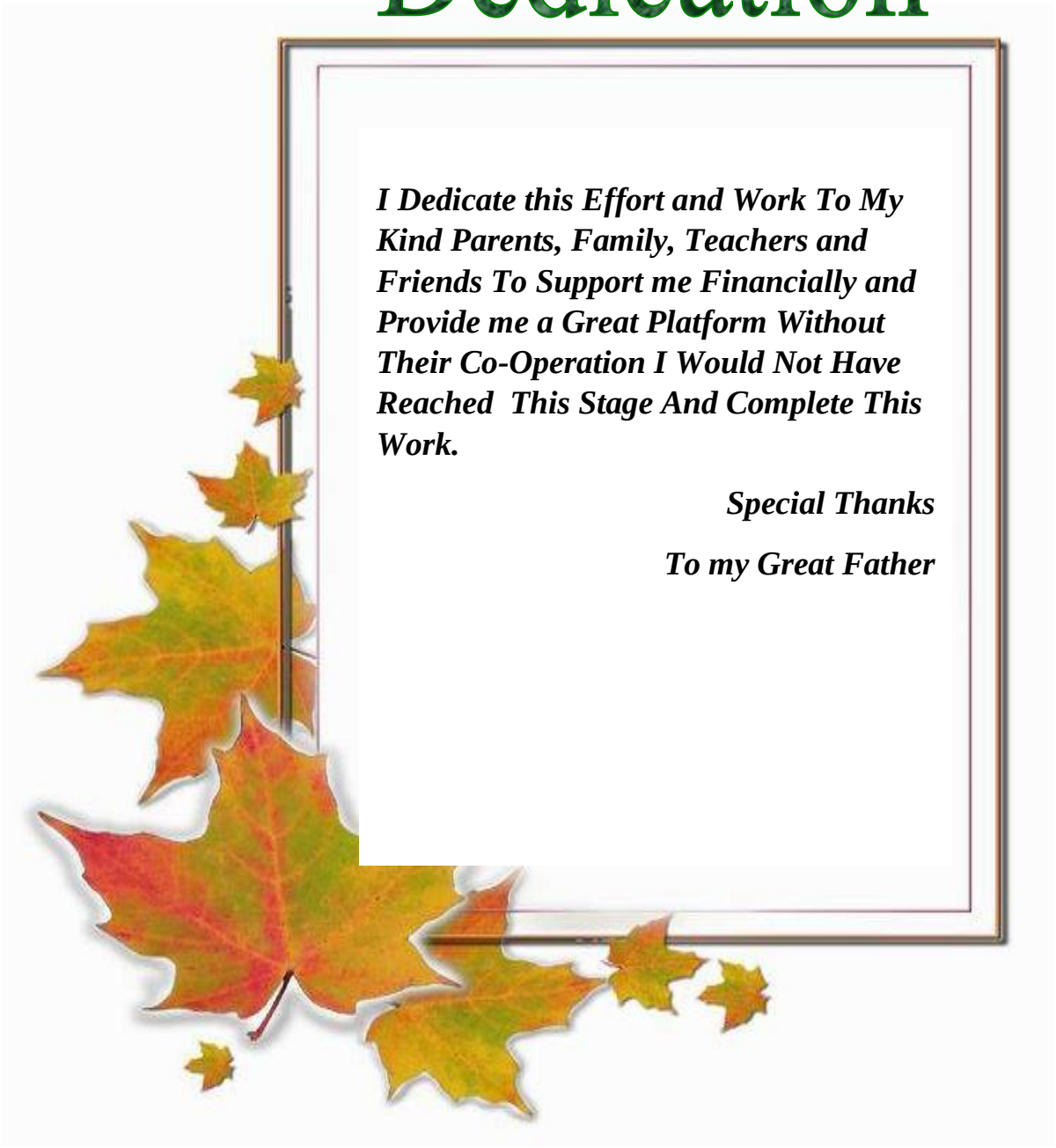
Bismillah Hirrahman Nirraheem.

In the name of Allah, the most gracious, the most beneficent, the most compassionate, the most merciful and may the peace and blessings be upon the messenger of Allah and his family and companions and whoever has been guided to the right path.

Sallallahu Ala Mu'hammad Sallallahu Alayhe Wasallam.

Dedication



I Dedicate this Effort and Work To My Kind Parents, Family, Teachers and Friends To Support me Financially and Provide me a Great Platform Without Their Co-Operation I Would Not Have Reached This Stage And Complete This Work.

Special Thanks

To my Great Father

TABLE OF CONTENTS

Serial No.	Title	Page No.
1	**Acknowledgements**	**i**
2	**List of Abbreviations**	**ii**
3	**List of Figures**	**iv**
4	**List of Tables**	**viii**
5	**General Abstract**	**ix**
6	**General Introduction**	**1**
7	**Aims and objectives**	**19**
8	**Chapter 1:** Comparative effects of different concentrations of Bisphenol A and its analogues bisphenol B, bisphenol F and bisphenol S on the epididymal sperms and testicular tissues of male Sprague Dewley rats.	
	I. Abstract	**21**
	II. Introduction	**22**
	III. Materials and Methods	**24**
	IV. Results	**30**
	V. Discussion	**38**
	VI. Conclusion	**39**

TABLE OF CONTENTS

Serial No.	Title	Page No.
9	**Chapter 2:** Assessment of bisphenol A and its analogues bisphenol B, bisphenol F and bisphenol S induced sub-chronic reproductive toxicity in male rats: a biochemical, morphological and histological study.	
	I. Abstract	**40**
	II. Introduction	**41**
	III. Materials and Methods	**43**
	IV. Results	**48**
	V. Discussion	**64**
	VI. Conclusion	**68**
10	**Chapter 3:** Long term chronic exposure to bisphenol A and its analogues bisphenol B, bisphenol F and bisphenol S on the reproductive system of male rats: possible mechanism of estrogen action.	
	I. Abstract	**69**
	II. Introduction	**71**
	III. Materials and Methods	**74**
	IV. Results	**77**
	V. Discussion	**103**
	VI. Conclusion	**106**

TABLE OF CONTENTS

Serial No.	Title	Page No.
11	**Chapter 4:** Comparative study on the endocrine-disrupting activity of bisphenol A and its safe substitutes bisphenol B, bisphenol F and bisphenol S: development effects on prenatal offspring of male rats.	
	I. Abstract	**107**
	II. Introduction	**108**
	III. Materials and Methods	**110**
	IV. Results	**114**
	V. Discussion	**136**
	VI. Conclusion	**139**
12	**Chapter 5:** From bisphenol A to its analogues bisphenol B, bisphenol F and bisphenol S in rats: a neonatal study on the sexual development of male rats.	
	I. Abstract	**141**
	II. Introduction	**142**
	III. Materials and Methods	**145**
	IV. Results	**148**
	V. Discussion	**178**
	VI. Conclusion	**181**

TABLE OF CONTENTS

13	General Discussion	**182**
14	General conclusion and future prospective	**187, 191**
15	References	**193**

Author's Declaration

I Mr. Asad Ullah herby state that my Ph.D thesis titled "**Are Structural Analogues to Bisphenol A Safe Alternatives? Comparative In-vitro and In-vivo Approaches to Study Reproductive Toxicity in Male Rats"** is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

At any time if my statement is found to be incorrect even after my Graduate the University has the right to withdraw my Ph.D. degree.

Asad Ullah

Date: _____

LIST OF TABLES

Table No	Title	Page No
1	Calculated physiochemical properties of bisphenol A and its analogues BPB, BPF and BPS.	**13**
2	Toxic effects of bisphenol A and its analogues as BPB, BPF and BPS.	**13**
3	*In vitro* effects of bisphenol A and its analogues BPB, BPF and BPS (1, 10 and 100 ng/ml) on antioxidant enzymes and testosterone concentrations in rat testis.	**31**
4	Mean ± SEM of rat sperm DNA damage in control and sperms incubated with different concentrations of BPA, BPB, BPF and BPS (1, 10, and 100 ug/L) for 2 hours.	**37**
5	Acute toxicity of different concentrations of bisphenol A and its analogues BPB, BPF and BPS (0, 5, 50, 300 and 2000 mg/kg) after single oral dose in female rats.	**51**
6	Effects of sub-chronic exposure of different concentrations of Bisphenol A and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) on the different parameters of rat testis.	**52**
7	Effects of sub-chronic exposure of different concentrations of bisphenol A and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) on the biochemical parameters of rats.	**53**
8	Effects of sub-chronic exposure to different concentrations of bisphenol A and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) on the testicular testosterone concentrations in rats.	**55**
9	Effects of sub-chronic exposure to different concentrations of bisphenol A and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) on the testis morphometry.	**58**
10	Effects of sub-chronic exposure to different concentrations of bisphenol A and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) on the epididymal caput morphometry.	**59**

LIST OF TABLES

Table No	Title	Page No
11	Effects of sub-chronic exposure to different concentrations of bisphenol A and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) on the epididymal cauda morphometry.	**60**
12	Effects of sub-chronic exposure to different concentrations of bisphenol A and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) in rats seminiferous tubules and number of different types of cells.	**61**
13	Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 μg/L) on body weight gain of rats.	**76**
14	Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 μg/L) on body and organs weight of rats.	**78**
15	Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 μg/L) on the organs weight of rats.	**79**
16	Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 μg/L) on oxidative stress in the testicular tissues of rats.	**81**
17	Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 μg/L) on plasma testosterone, estradiol, LH and FSH concentrations in rats.	**84**
18	Effect of chronic exposure of different concentrations of BPA and its alternatives BPB, BPF and BPS (5, 25 and 50 μg/L) on sperm parameters and sperm number in epididymis of rats.	**85**
19	Effect of chronic exposure of different concentrations of BPA and its alternatives BPB, BPF and BPS (5, 25 and 50 μg/L) on planimetry of testis in rats.	**90**

LIST OF TABLES

Table No	Title	Page No
20	Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 μg/L) on number of different cell types in the testis of rats.	**92**
21	Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 μg/L) on planimetry of caput epididymis in rats.	**98**
22	Effect of chronic exposure of different concentrations of BPA and its analogues BPS, BPF and BPS (5, 25 and 50 μg/L) on planimetry of cauda epididymis in rats.	**99**
23	Effect of different concentrations (5, 25 and 50 μg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on offspring.	**110**
24	Effect of different concentrations (5, 25 and 50 μg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on organ and body weights of offspring on PND 16.	**112**
25	Effect of different concentrations (5, 25 and 50 μg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on organ of offspring on PND 16.	**113**
26	Effect of different concentrations (5, 25 and 50 μg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on organ and body weights of offspring on PND 80.	**114**
27	Effect of different concentrations (5, 25 and 50 μg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on organ and body weights of offspring on PND 80.	**115**
28	Effect of different concentrations (5, 25 and 50 μg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PD 1 on daily sperm production (DSP) and sperm in different parts of epididymis of rats on postnatal day (PND 80).	**117**

LIST OF TABLES

Table No	Title	Page No
29	Effect of different concentrations (5, 25 and 50 μg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on testis histology of rats on PND 80.	**120**
30	Effect of different concentrations (5, 25 and 50 μg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on caput epididymis histology of rats on PND 80.	**121**
31	Effect of different concentrations (5, 25 and 50 μg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on cauda epididymis histology of rats on PND 80.	**122**
32	Effect of different concentrations (5, 25, and 50 μg/L) of BPA and its analogies BPB, BPF and BPS exposure from GD 1 to PD 1 on plasma testosterone and estrogen concentrations in rats on PD 80.	**123**
33	Effect of neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS on body weight gain in adult rats.	**143**
34	Effect of neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS on organ weight in adult rats.	**145**
35	Effect of neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS on organ weight in adult rats.	**147**
36	Effect of neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS on organ weight in adult rats.	**148**
37	Effect of neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS on the antioxidants profile in adult rats.	**152**

LIST OF TABLES

Table No	Title	Page No
38	Effect of neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS on plasma testosterone, estradiol, Luteinizing hormone and Follicle Stimulating hormone concentrations in adult rats.	**153**
39	Daily sperm production (DSP), Sperm motility and sperm count parameters in the testis and different parts of epididymis in adult rats after neonatal exposure to different concentrations of Bisphenols as BPA, BPB, BPF and BPS.	**156**
40	Histopathological study of testicular tissues of adult rats after neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS.	**159**
41	Seminiferous tubules number and different types of cell in the testis of adult rats after neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS.	**163**
42	Results of the caput epididymis morphometry in the adult rats after neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS.	**165**
43	Results of the cauda epididymis morphometry in the adult rats after neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS.	**166**

List of Figures

Figure #	Title	Page No
1	Routes of endocrine disruptors (EDCs) release in to the environment and their toxic effects on human health.	3
2	Chemical structures of bisphenol A (BPA) and its analogues bisphenol B (BPB), bisphenol F (BPF) and bispheonol S (BPS) used in the present study.	9
3	Mechanism of action of bisphenol A and its analogues BPB, BPF and BPS through production of oxidative stress in the body.	11
4	Mechanism of action of bisphenol A and its analogues BPB, BPF and BPS at different hormonal and receptor level.	15
5	*In vitro* effect of BPA, BPB, BPF and BPS on SOD activity in rat sperms after 2 hours of incubation. SOD activity measured in control and BPA, BPB, BPF and BPS (1, 10, and 100 µg/L) treated groups. Results are expressed as mean ± SEM (n = 7 for each condition) and presented as SOD (mU/10^8 cells). Significant results (P < 0.05 and P < 0.01) are indicated: *, ** versus control.	34
6	Effects of BPA, BPB, BPF and BPS on TBARS in rat sperm after 2 hours of incubation *in vitro*. TBARS measured in control and BPA, BPB, BPF and BPS (1, 10, and 100 µg/L) treated rat sperm groups are expressed as mean ± SEM (n = 7 for each condition) and presented as TBARS (nmol malonaldehyde/10^8 cells). Significant results (P < 0.01 and P < 0.01) are indicated: *, **versus control.	35
7	Effects of BPA and its analogues BPB, BPF and BPS on ROS in rat sperm after 2 hours of incubation *in vitro*. ROS measured in control and BPA, BPB, BPF and BPS (1, 10, and 100 µg/L) treated rat sperm groups are expressed as mean ± SEM (n = 7 for each condition) and presented as ROS (units/10^8 cells). Significant results (p < 0.05) are indicated: *versus control	36

List of Figures

Figure #	Title	Page No
8	Photomicrograph from testicular tissue showing (A) control; with compact arrangement of seminiferous tubules with thick epithelial height (Line without arrow head) and elongated spermatids (arrow) (B, C and D); BPA (5, 25 and 50 mg/kg/day) treated groups presenting seminiferous tubules with epithelium (line without arrow head) and spermatids (white arrow): (E, F and G) BPB (5,25 and 50 mg/kg/day) treated groups presenting seminiferous tubules with epithelium (line without arrow head) and elongated spermatids (white arrow) (H, I and J) BPF (5, 25 and 50 mg/kg/day) treated groups presenting seminiferous tubules with epithelium (line without arrow head) and elongated spermatids (white arrow); (K, L and M) BPS (5, 25 and 50 mg/kg/day) treated groups presenting seminiferous tubules with epithelium (line without arrow head) and spermatids (white arrow). H&E (x40)	62
9	Photomicrograph of cross section of caput epididymis from: (A) Control; showing normal morphology with compactly arranged tubules and thick epithelium, lumen filled with sperm (arrow), (B, C and D) BPA (5, 25, 50 mg/kg/day) treated groups presenting tubules with epithelium and lumen with sperms (white arrow), (E, F and G) BPB (5, 25 and 50 mg/kg/day) treated presenting caput tubules with epithelium and sperm in the lumen, (H, I and J) BPF (5, 25, 50 mg/kg/day) treated groups presenting tubules with epithelium and sperm in the lumen, (K, L and M) BPS (5, 25 and 50 mg/kg/day) treated groups presenting tubules with epithelium and sperm in the lumen (white arrow).H&E (x40)	63
10	Photomicrograph of cross section of cauda epididymis from: (A) Control; showing normal morphology of cauda epididymis with tubules and thick epithelium, lumen filled with sperms (arrow), (B, C and D) BPA (5, 25, 50 mg/kg/day) treated groups presenting cauda tubules with epithelium and sperms in the lumen (white arrow): (E, F and G) BPB (5, 25 and 50 mg/kg/day) treated groups presenting cauda tubules with sperm in the lumen (white arrow). (H, I and J) BPF (5, 25 and 50 mg/kg/day) treated groups presenting cauda tubules with sperm in the lumen. (K, L and M) BPS (5, 25 and 50 mg/kg/day) treated presenting cauda tubules with sperm in the lumen. (white arrow).H&E (x40)	64

List of Figures

Figure #	Title	Page No
11	Photomicrograph from testicular tissue showing (A) control; having thick epithelium with normal spermatogonia (SP), Round spermatids (RS), Elongated spermatids (ES) and filled lumen with sperm (B, C and D); BPA (5, 25 and 50 µg/L) treated presenting seminiferous tubules with epithelium (line without arrow head) and spermatids (white arrow); (E, F and G) BPB (5, 25 and 50 µg/L) treated presenting seminiferous tubules with epithelium (line without arrow head) and elongating spermatids (white arrow); (H, I and J) BPF (5, 25 and 50 µg/L) treated presenting seminiferous tubules with epithelium (line without arrow head) and elongating spermatids (white arrow); (K, L and M) BPS (5, 25 and 50 µg/L) treated presenting seminiferous tubules with epithelium (line without arrow head) and spermatids (white arrow). H&E (x40)	88
12	Photomicrograph of caput epididymis tissue showing (A) control; with compact arrangement of caput tubules with sperm filled lumen (B) BPA (5 µg/L) exposed group, presenting normal caput tubules like in the control (C), BPA (25 µg/L) exposed group showing seminiferous tubules with less number of sperm in the lumen (arrow) and (D) BPA (50 µg/L) exposed group presenting caput tubules with empty lumen (arrow). Similarly, (E) BPB (5 µg/L) exposed group, presenting normal caput tubules, (F) BPB (25 µg/L) exposed group showing less number of sperms in the lumen, (G) BPB (50 µg/L) exposed group showing less number of sperms and empty lumen (arrow). (H) BPF (5 µg/L) exposed group, presenting normal caput tubules, (I) (25 µg/L) exposed group showing seminiferous tubules with less number of sperm in the lumen (arrow) and (J) BPF (50 µg/L) exposed group showing less number of sperms and empty lumen (Arrow). K, L BPS (5, 25 µg/L) exposed groups showing caput tubules with less number of sperms in the lumen and (M) BPS (50 µg/L) exposed group presenting less number of sperms and empty lumen. H&E (x40)	94

List of Figures

Figure #	Title	Page No
13	Photomicrograph of cauda epididymis tissue showing (A) control; with compact arrangement of cauda tubules with sperm filled lumen (B) BPA (5 µg/L) exposed group, presenting normal caput tubules like in the control (C) BPA (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (D) BPA (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. Similarly, (E) BPB (5 µg/L) exposed group, presenting normal caput tubules like in the control (F) BPB (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (G) BPB (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. Likewise, (H) BPF (5 µg/L) exposed group, presenting normal caput tubules like in the control (I) BPF (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (J) BPF (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. In the same way, (K) BPS (5 µg/L) exposed group, presenting normal caput tubules like in the control (L) BPS (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (M) BPS (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. H&E (x40)	96
14	Photomicrograph from testicular tissue showing (A) control; having thick epithelium with normal spermatogonia (SP), Round spermatids (RS), Elongated spermatids (ES) and filled lumen with sperm (B, C and D); BPA (5, 25 and 50 µg/L) treated presenting seminiferous tubules with epithelium (line without arrow head) and spermatids (White arrow); (E, F and G) BPB (5, 25 and 50 µg/L) treated presenting seminiferous tubules with epithelium (line without arrow head) and elongating spermatids (white arrow); (H, I and J) BPF (5, 25 and 50 µg/L) treated presenting seminiferous tubules with epithelium (line without arrow head) and elongating spermatids (white arrow); (K, L and M) BPS (5, 25 and 50 µg/L) treated presenting seminiferous tubules with epithelium (line without arrow head) and spermatids (white arrow). H&E (40x)	125

List of Figures

Figure #	Title	Page No
15	Photomicrograph of caput epididymis tissue showing (A) control; with compact arrangement of caput tubules with sperm filled lumen (B) BPA (5 µg/L) exposed group, presenting normal caput tubules like in the control (C), BPA (25 µg/L) exposed group showing seminiferous tubules with less number of sperm in the lumen (arrow) and (D) BPA (50 µg/L) exposed group presenting caput tubules with empty lumen (arrow). Similarly, (E) BPB (5 µg/L) exposed group, presenting normal caput tubules, (F) BPB (25 µg/L) exposed group showing less number of sperms in the lumen, (G) BPB (50 µg/L) exposed group showing less number of sperms and empty lumen (arrow). (H) BPF (5 µg/L) exposed group, presenting normal caput tubules, (I) (25 µg/L) exposed group showing seminiferous tubules with less number of sperm in the lumen (arrow) and (J) BPF (50 µg/L) exposed group showing less number of sperms and empty lumen (arrow). K and L BPS (5 and 25 µg/L) exposed groups showing caput tubules with less number of sperms in the lumen and (M) BPS (50 µg/L) exposed group presenting less number of sperms and empty lumen. H&E (40x)	127
16	Photomicrograph of cauda epididymis tissue showing (A) control; with compact arrangement of cauda tubules with sperm filled lumen (B) BPA (5 µg/L) exposed group, presenting normal caput tubules like in the control (C) BPA (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (D) BPA (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. Similarly, (E) BPB (5 µg/L) exposed group, presenting normal caput tubules like in the control (F) BPB (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (G) BPB (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. Likewise, (H) BPF (5 µg/L) exposed group, presenting normal caput tubules like in the control (I) BPF (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (J) BPF (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. In the same way, (K) BPS (5 µg/L) exposed group, presenting normal caput tubules like in the control (L) BPS (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (M) BPS (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. H&E (40x)	129

List of Figures

Figure #	Title	Page No
17	Photomicrograph from testicular tissue showing (A) control; having thick epithelium with normal spermatogonia (SP), Round spermatids (RS), Elongated spermatids (ES) and filled lumen with sperm (B, C and D); BPA (5, 25 and 50 mg/kg/day) treated presenting seminiferous tubules with epithelium (line without arrow head) and spermatids (white arrow); (E, F and G) BPB (5,25 and 50 mg/kg/day) treated presenting seminiferous tubules with epithelium (line without arrow head) and elongating spermatids (white arrow); (H, I and J) BPF (5, 25 and 50 mg/kg/day) treated presenting seminiferous tubules with epithelium (line without arrow head) and elongating spermatids (white arrow); (K, L and M) BPS (5, 25 and 50 mg/kg/day) treated presenting seminiferous tubules with epithelium (line without arrow head) and spermatids (white arrow). H&E (40X)	160
18	Photomicrograph of caput epididymis tissue showing (A) control; with compact arrangement of caput tubules with sperm filled lumen (B) BPA (5 mg/kg/day) exposed group, presenting normal caput tubules like in the control (C), BPA (25 mg/kg/day) exposed group showing seminiferous tubules with less number of sperm in the lumen (arrow) and (D) BPA (50 µg/L) exposed group presenting caput tubules with empty lumen (arrow). Similarly, (E) BPB (5 mg/kg/day) exposed group, presenting normal caput tubules, (F) BPB (25 mg/kg/day) exposed group showing less number of sperms in the lumen, (G) BPB (50 mg/kg/day) exposed group showing less number of sperms and empty lumen (arrow). (H) BPF (5 mg/kg/day) exposed group, presenting normal caput tubules, (I) (25 mg/kg/day) exposed group showing seminiferous tubules with less number of sperm in the lumen (arrow) and (J) BPF (50 mg/kg/day) exposed group showing less number of sperms and empty lumen (arrow). K, L BPS (5, 25 mg/kg/day) exposed groups showing caput tubules with less number of sperms in the lumen and (M) BPS (50 mg/kg/day) exposed group presenting less number of sperms and empty lumen. H&E (40X)	167

List of Figures

Figure #	Title	Page No
19	**Photomicrograph of cauda epididymis tissue showing (A) control; with compact arrangement of cauda tubules with sperm filled lumen (B) BPA (5 mg/kg/day) exposed group, presenting normal caput tubules like in the control (C) BPA (25 mg/kg/day) exposed group, presenting cauda tubules with sperm filled lumen (D) BPA (50 mg/kg/day) exposed group presenting cauda tubules with less sperm in the lumen. Similarly, (E) BPB (5 mg/kg/day) exposed group, presenting normal caput tubules like in the control (F) BPB (25 mg/kg/day) exposed group, presenting cauda tubules with sperm filled lumen (G) BPB (50 mg/kg/day) exposed group presenting cauda tubules with less sperm in the lumen. Likewise, (H) BPF (5 mg/kg/day) exposed group, presenting normal caput tubules like in the control (I) BPF (25 mg/kg/day) exposed group, presenting cauda tubules with sperm filled lumen (J) BPF (50 mg/kg/day) exposed group presenting cauda tubules with less sperm in the lumen. In the same way, (K) BPS (5 mg/kg/day) exposed group, presenting normal caput tubules like in the control (L) BPS (25 mg/kg/day) exposed group, presenting cauda tubules with sperm filled lumen (M) BPS (50 mg/kg/day) exposed group presenting cauda tubules with less sperm in the lumen. H&E (40X)**	**169**
20	***In vitro* exposure of different concentrations of BPA and its analogues BPB, BPF and BPS on the sperm and testicular tissues of adult male rats. BPA and its analogues exposure reduced testosterone secretions and induced oxidative stress**	**181**
21	***In vivo* sub-chronic and chronic effects of different concentrations of BPA, BPB, BPF and BPS on the different parameters of hypothalamo-pitutary-testicular (HPT) axis of male rats. The above schematic representation show that BPA and its analogues BPB, BPF and BPS exposure in both sub chronic and chronic study can alter different hormones, lead to oxidative stress in the reproductive tissues and can also cause morphological changes in the number of different cells present inside the testis and epididymis**	**182**
22	**Schematic representation showing BPA, BPB, BPF and BPS effects on the development of male reproductive system in rats.**	**183**

	Pre-natal and neo-natal treated rats showed alterations in the reproductive hormones and also an increase was observed in the oxidative stress in the testicular tissues of male rats	

LIST OF ABBREVIATIONS

BPA	**Bisphenol A**
BPB	**Bisphenol B**
BPF	**Bisphenol F**
BPS	**Bisphenol S**
ANOVA	**Analysis of variance**
DEPPD	**N, n-diethyl-para-phenylenediamine**
DES	**Diethylstilbestrol**
DMEM	**Dulbecco`s modified eagle`s medium**
ELISA	**Enzyme linked immuno sorbant assay**
EPA	**Environmental protection agency**
ERα	**Estrogen nuclear receptor alpha**
GABA	**Gamma-amino-butyric acid**
GnRH	**Gonadotropin-releasing hormone**
GSI	**Gonad somatic index**
hERα	**Human estrogen receptor alpha**
HOS	**Human osteosarcoma cell line**
WHO	**World health organization**
TBARS	**Thiobarbituric acid reactive substances**
SOD	**Superoxide dismutase**
ROS	**Reactive oxygen species**
PPARG	**Peroxisome proliferator-activated receptor-γ**
POD	**Peroxidase**

PND	**Postnatal day**
PBS	**Phosphate buffer saline**
PBMCs	**Peripheral blood mononuclear cells**
OECD	**Organization for economic co-operation and development**
LPO	**Lipid peroxidation**
LH	**Luteinizing hormone**
JNK	**C-jun n-terminal kinase**
HSL	**Hormone-sensitive lipase**
HRP	**Horseradish peroxidase**
HPGA	**Hypothalamic pituitary gonadal axis**
HOS	**Human osteosarcoma cell line**
EDC	**Endocrine disrupting chemical**
ECHA	**European chemicals agency**
BAC	**Biological activated carbon**
BaP	**Benzo[a]pyrene**
BDL	**Below detection limits**
COA	**Chemical advanced oxidants**
DE	**Diethylether**
DEC CW	**Department of Environment Climate Change and Water**
DMSO	**Dimethylsulfoxide**
ER	**Estrogen receptor**
ERBA	**Estrogen receptor binding assay**
GC-MS	**Gas chromatography mass spectrometry**

H_2S0_4	**Sulphuric acid**
HAH	**Halogenated aromatic hydrocarbon**
hER	**Human estrogen receptor**
αE2	**17 α-estradiol**

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GENERAL ABSTRACT

Background: Environmental health has emerged recently from observing the low outcomes of reproductive success of wildlife, birds and fish population in relation of their exposure to industrial chemicals. The intensive use of pesticides in agriculture and exposure of toxic chemicals which directly or indirectly lead to alterations in the reproductive functions of both human and wildlife to the concept of endocrine disrupting chemicals (EDCs). In the modern day EDCs list bisphenol A (BPA) has taken a prominent place. BPA is mainly used these days in the manufacturing of plastic bottles, plastic utensils, food containers, baby toys, feeding bottles and medical equipment. Studies have also shown that apart of wide range of useful applications BPA has hazardous mode of action on many systems of the body. Due to its wide range of toxic actions BPA has been banned in many countries including the European Union (EU) in several daily use items. This ban has led to the introduction of many BPA analogues which are considered to be safer than BPA and are these days used in the production of many daily use items. Bisphenol B (BPB), bisphenol F (BPF) and bisphenol S (BPS) are among the top of the safe BPA list. These all analogues consist of two phenol groups attached with a carbon of any other chemical bridge. Since these structurally similar compounds to BPA are expected to have similar or even stronger toxicological effects on humans and wildlife. Due to the ban of BPA in some other countries the production of these BPA analogues is at rise and is expected that this production is going to increase by double in the future. On the other hand, BPA analogues have already registered their presence in many environmental compartments as well as food, beverages and drinking water which has not only increased the risk of exposure to occupational and also general population. In the recent years studies have also shown that some of these analogues have shown estrogenic activity, potentials of inducing oxidative stress and as well as anti-androgenic effects in many experimental animal studies. Although the toxicity of BPA has been studied in great detail regarding reproductive functions in both mammalian and non-mammalian species though data regarding BPA analogues is scare. This brings the need for making a comprehensive data bank on the so called safe analogues of BPA. The main purpose of the present set of studies is to assess both *in-vitro* and *in-vivo* effects of these analogues on the sperm and testicular tissues of male rats. In this regard another set of sub-chronic study was done to compare the reproductive toxicity in male rats after exposure to the BPA analogues. In the last but not the least another set of experiments was carried out to understand the potential effects of

BPA and its analogues BPB, BPF and BPS on the development of male reproductive system in both prenatal and neonatal male rats.

Materials and methods: BPA and its analogues BPB, BPF and BPS stock solutions were prepared in ethanol and were later diluted in distilled water and the final concentration in every stock solution was less than 0.5% ethanol. In our first experiment we incubated male adult rats' testicular tissues and sperms in different concentrations (0, 1, 10 and 100 µg/L) of BPA, BPB, BPF and BPS for two hours. The temperature was maintained as 37 ^{0}C, CO_2 was 5 % and air was 95 %. Oxidative stress in the reproductive tissues was determined through antioxidant enzymes and hormonal concentration was determined through Enzyme Linked Immuno Sorbant assay (ELISA). In the second sub-chronic experiment adult male rats were treated with different concentrations of BPA and its analogues BPB, BPF and BPS for 28 days. The third experiment was chronic experiment where adult male rats were again exposed to different concentrations of BPA and its analogues BPB, BPF and BPS through drinking water for a period of 48 weeks. After the completion of sub-chronic and chronic experiment animals were euthanized and different biochemical, hormonal and histological tests were carried out. In the next set of experiments, effects of BPA and its analogues BPB, BPF and BPS on the development of male gonads was assessed by exposing the animals to different concentrations of BPA and its analogues BPB, BPF and BPS during pre-natal and neonatal period of development. In the fourth experiment female pregnant rats were exposed to different concentrations of BPA and its analogues BPB, BPF and BPS from pregnancy day 1 (PD1) to PD 10. The born pups were checked for any alterations in the early sexual development and any reproductive complacency during the adulthood. In the last and fifth experiment male newborn pups were exposed to different concentrations of BPA and its analogues BPB, BPF and BPS from Post natal day 1(PND 1) to PND 10 and early sexual development or any alteration in the reproductive functions were checked throughout the experimental period. Biochemical, hormonal and histological tests were carried out of different reproductive organs.

Results: The results of the *in-vitro* study showed that BPA and its analogues BPB, BPF and BPS led to oxidative stress in the testicular tissues and sperms and antioxidant enzyme activity was also increased after the treatment with BPA and its analogues BPB, BPF and BPS. The higher treatment groups caused lipid peroxidation, increased DNA fragmentation and affected

superoxide dismutase levels in the spermatozoa of male rats. BPA and its analogues BPB, BPF and BPS higher dose groups also reduced the testosterone concentration in the rat testis. Sub-chronic and chronic *in vivo* studies on the other hand showed reduced plasma and intra-testicular testosterone, plasma luteinizing hormone (LH) and follicle stimulating hormone (FSH) concentrations in the groups with higher treatment of BPA and its analogues BPB, BPF and BPS. Antioxidant enzymes activity significantly reduced after exposure to BPA and its analogues BPB, BPF and BPS when compared to the control. Histopathological results revealed alterations in the normal morphology of testicular tissues as compared to control. Histological observations showed significant reduction in the epithelial height of the testis along with disrupted spermatogenesis. Other prominent observations were empty lumen of the seminiferous tubules and caput region of the epididymis. Daily sperm production (DSP), sperm motility and oxidative stress markers in the testis in the chronic and sub-chronic groups after treatment with different concentrations of BPA and its analogues BPB, BPF and BPS showed disturbed hormonal concentrations and antioxidant enzymes. The results of the pre-natal and post-natal exposure of different concentrations of BPA and its analogues BPB, BPF and BPS showed no prominent sign of early puberty and development of sexual organs. On the other hand, significant decrease was observed in the gonadosomatic index (GSI) and organs weight when higher doses treatment groups of BPA and its analogues BPB, BPF and BPS were compared to the control. Significant reduction was observed in the hormonal concentrations of testosterone, LH and FSH when compared to the control. Significant reduction was observed in the DSP and sperm number in the caput and cauda region in the highest treatment groups of BPA and its analogues BPB, BPF and BPS as compared to control. Histopathological analysis showed that BPA and its analogues BPB, BPF and BPS treatment reduced epithelial height and sperm arrest as compared to the control group; there were also alterations observed in the morphology of different cells in the reproductive organs of male rats after exposure to BPA and its analogues.

Conclusions: BPA and its analogues BPB, BPF and BPS induced oxidative stress in the reproductive organs and also showed endocrine disrupting potentials and toxicological results in the *in-vivo* and *in-vitro* studies. The results of the present study also showed that exposure to BPA and its analogues BPB, BPF and BPS in the pre-natal and post-natal life not only lead to toxicity in the development of reproductive organs but also lead to changes in the reproductive organs which cannot be reversed in the adult life. BPA and its analogues BPB, BPF and BPS

also resulted in adverse structural and functional changes in the reproductive system by inhibiting the cell defense system. These effects also lead to suppression of gonadotropins, anti-androgenic and estrogenic mode of actions which can cause deleterious alterations in the reproductive tissues which can harm the normal fertility of individuals. After the present study results the question arise that whether this shift towards the analogues of BPA is safe or more life threatening than BPA exposure? The present data results are raising concerns that there shall be comprehensive data bank on the comparative analysis of BPA and its analogues made on both mammals and non-mammalian species.

Chapter # 1

Comparative effects of different concentrations of bisphenol A and its analogues bisphenol B, bisphenol F and bisphenol S on the epididymal sperms and testicular tissues of male Sprague Dawley rats

Chapter # 2

Assessment of bisphenol A and its analogues bisphenol B, bisphenol F and bisphenol S induced sub-chronic reproductive toxicity in male rats: a biochemical and histological study

Chapter # 3

Long term chronic exposure to Bisphenol A and its analogues bisphenol B, bisphenol F and bisphenol S on the reproductive system of male rats: possible mechanism of estrogen mode of action

Chapter # 4

Comparative study on the endocrine-disrupting activity of bisphenol A and its analogues bisphenol B, bisphenol F and bisphenol S: Developmental effects on prenatal offspring of Sprague Dawley rats

Chapter # 5

From bisphenol A to its analogues bisphenol B, bisphenol F and bisphenol S in rats: A neonatal study on the sexual development of male rats

GENERAL INTRODUCTION

Endocrine Disrupting Chemicals (EDCs) are exogenous compounds, which interact with the animal's or human's endocrine system by stimulating, blocking and repressing the normal homeostasis. EDCs can inhibit the release and production of hormones leading to disturbed endocrine system which result in metabolic problems or lead to the promotions of clinical disorders in human (Toro-Vélez *et al.*, 2016). EDCs have led to many problems as the weak male reproductive system, testicular and prostate cancer, affected immune system, increase in the breast tissues and many more problems associated with the reproductive system (Ahmed *et al.*, 2017). Until now there is a big list of around 800 chemicals which are considered as EDC because of their potential endocrine disruption. According to literature EDCs can be classified into two categories depending on their origin of either natural or synthetic. The first category includes natural compounds as genistein and phytoestrogens which are mainly found in the human and animal food. Whereas the second category includes chemicals used in the industry as polybrominated biphenyls (PBBs), dioxins, pesticides, phthalates, plasticizers, polychlorinated biphenyls (PCBs) and fungicides (Kabir *et al.*, 2015). Among these all EDCs one of them is a very well-known endocrine disruptor called bisphenol A (BPA).

Effect of EDCs on reproduction

EDCs are involved in disturbing the normal physiology of the reproductive system. *In vitro* and *in vivo* results have shown testicular and ovarian abnormalities with an increase in the occurrence of breast, prostate and testicular cancer (Miller and Sharpe, 1998, Toppari *et al.*, 1996). Environmental pollutants are also observed to play key role in the sexual defects of wildlife species (Maffini *et al.*, 2006). Among the main reproductive problems, testicular carcinoma, testicular dysgenesis syndrome and poor quality of semen are the main associated issues with the affected endocrine system due to environmental pollutants (Skakkebæk *et al.*, 2001, Carlsen *et al.*, 1992). A study from Belgium of 120 young girls of the general population was observed to have complacencies in the development of reproductive stage due to exposure with polychlorinated bisphenols (PCBs) (Staessen *et al.*, 2001). There have also been studies which have shown that commonly exposed EDCs leach from baby bottles, water carboys, household materials, and packaging materials which have led to complacencies with reproductive malfunctioning of children (Biles *et al.*, 1997). In female rat, accidental exposure to BPA from

the plastic cage and water bottle caused aneuploidy and disturbance in the oocytes formation (Hunt *et al.*, 2003, Ikezuki *et al.*, 2002). In another study, it was observed that the risks of mischarge increase in women exposed to BPA (Hassold and Hunt, 2001, Sugiura-Ogasawara *et al.*, 2005).

EDCs are involved in effecting the normal physiology of the body by genomic, non-genomic, receptor-linked or non-receptor linked pathways. It has been observed that oxidative stress due to reactive oxygen species and generation of free radicals is the main cause of many bodily diseases not only in human but also wildlife due to exposure to EDCs (Nadal *et al.*, 2001, Saeidnia and Abdollahi, 2013, Swedenborg *et al.*, 2009).

EDCs damage the normal physiology of the body by causing cancer, reproductive problems, and growth abnormalities (Sanderson, 2006). Cytochrome 450 (CYP450) is the main component in the biosynthesis of different organs like adipose tissues, adrenal gland, brain, placenta, ovaries and testes (Fent and Stegeman, 1991). EDCs have been the main inhibitors of CYP450 which lead into "imposex (penis development in females)" by inhibiting the aromatase in females (Fent, 2003). Human and animals are at great risk due to exposure to these chemicals especially by oral and dermal route. EDCs like BPA and its family have been observed to have damaging effects on the central nervous system, thyroid, and reproduction. Bisphenols (BPA and its analogues) are mainly involved in affecting the endocrine hormones, ovarian fluids and endocrine disruption in the reproductive organs (Rogers *et al.*, 2013).

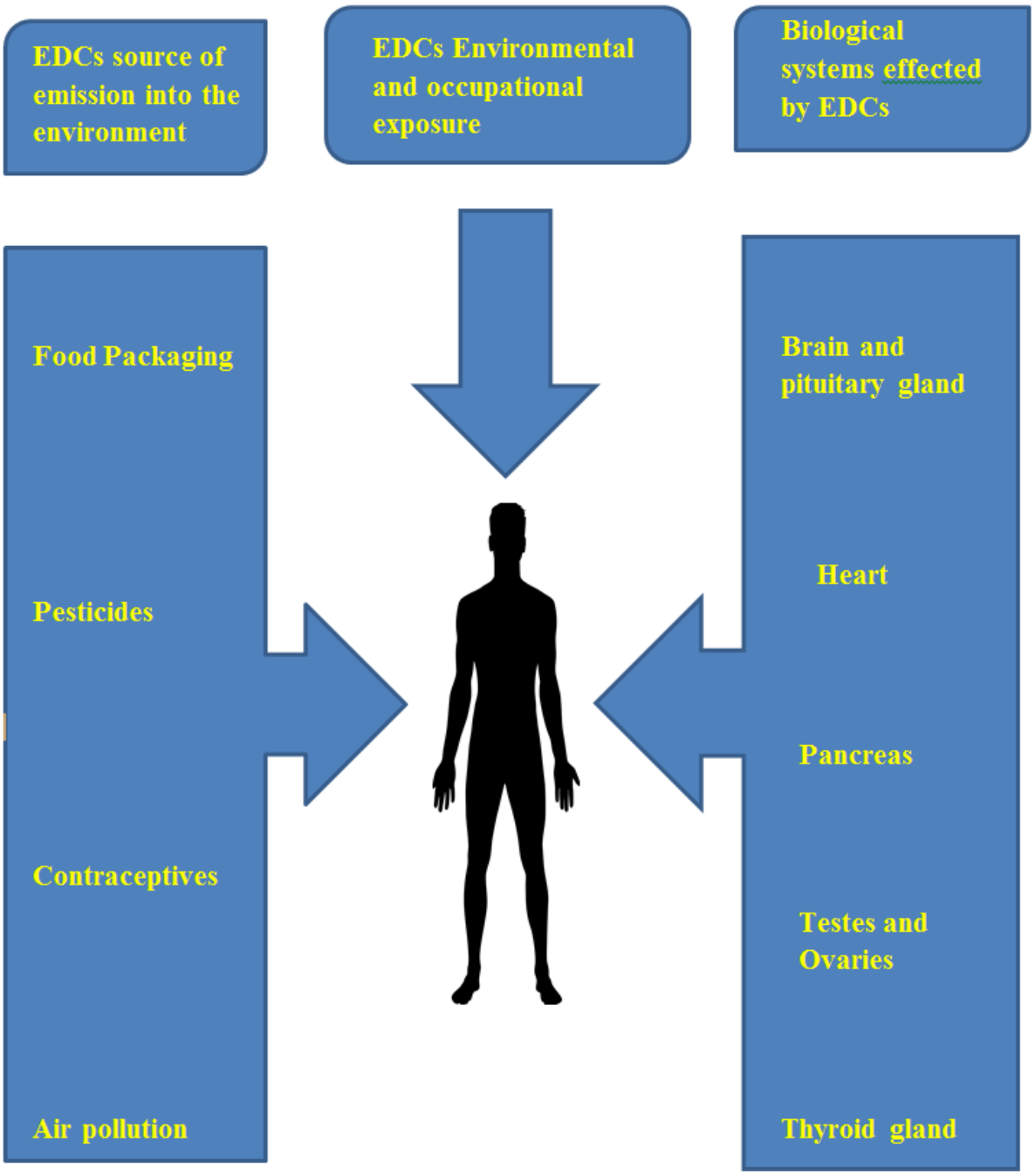

Fig 1. Routes of EDCs release into the environment and their toxic effects on human health

Bisphenol A (BPA)

BPA has had a very long history in the industrial world as it is a monomer produced in large quantity for the production of epoxy resins and polycarbonate plastics. BPA is used in the manufacturing of food cans, baby bottles and water pipes (Rubin, 2011). It is found in many consumer products these days such as dental composites, metal cans inside linings, thermal paper, beverage containers and food. Due to its high use in many consumer products BPA can leach from many products under acidic/basic conditions and high temperature showing many sources of human and animals exposure mostly via inhalation, dermal routes and digestion (Vandenberg *et al.*, 2010a, Vandenberg *et al.*, 2010b). Thus the health concerns regarding BPA are increasing due to its widespread human exposure (Calafat *et al.*, 2008, Qi and Zhang, 2011).

BPA can antagonize and mimic endogenous hormones by disturbing the endocrine function (Takayanagi *et al.*, 2006). As an environmental estrogen, it has high binding affinities for estrogen receptors (ER α and β) (Thomas and Dong, 2006). BPA through mimicking with the normal endocrine system influence normal differentiation and maturation process during embryonic development leading to chronic disease development such as obesity, type 2 diabetes and metabolic syndrome through the life course (Bateson *et al.*, 2004).

Health risks related to BPA exposure

BPA phenolic structure allows it to interact with estrogen receptors (ER) and act as agonist or antagonist via signaling pathways of endocrine system (Molina-Molina *et al.*, 2013). Therefore, it has been able to play role in the pathogenesis of many endocrine disorders including female and male fertility and many metabolic disorders and hormone-dependent tumors such as prostate and breast cancer (Diamanti-Kandarakis *et al.*, 2009). There are many routes of BPA exposure such as oral, transdermal and by inhalation and BPA main sources of exposure include healthcare equipment, thermal papers, toys, food packaging and indoor dust and infant feeding bottles (Geens *et al.*, 2012a). Among the main sources of BPA exposure, there is quite a big contribution of canned food stored in boxes either made or coated with BPA, meat and eggs of animals exposed to water with BPA (Huang *et al.*, 2012, Van Landuyt *et al.*, 2011, Oldring *et al.*, 2014). From the total produced BPA annually approximately 9% is used for the coating and lining of cans and other food packaging. Heating the cans for sterilization and food preparation leak BPA into the canned food from the epoxy coating and packaging which increase the potentials of BPA dietary exposure (Rubin, 2011, Kang and Kondo, 2003). During the

sterilization food higher concentration fat content food and lower pH have higher migration and concentration of BPA after being processed (Goodson *et al.*, 2004, Kang and Kondo, 2003, Munguia-Lopez *et al.*, 2002). The concentration of BPA has also been observed high in food stored in polycarbonate plastics especially during microwave cooking and heating (Viñas *et al.*, 2010). BPA exposure among children and infants may be high due to the common items containing BPA which are often taken by children into the mouth and inhaled (Hanaoka *et al.*, 2002, Geens *et al.*, 2012b).

Reasons for banning bisphenol A (BPA)

The demand in the manufacturing of BPA large quantity started after the discovery of Bayer and General Electric who found that BPA forms hard plastics called polycarbonate which was used in manufacturing of food packaging, baby and drinking bottles (Vogel, 2009). Which further enhanced the large production of BPA despite its synthetic estrogen nature (Rubin, 2011).

Among 309 environmental chemicals which have the ability to bind with endocrine mediated signaling, BPA is considered to be on 3rd highest priority index (Reif *et al.*, 2010). Later on several studies showed that BPA has effects on the brain and prostate gland in children (Shelby, 2008, Calafat *et al.*, 2008, Le *et al.*, 2008, Carwile *et al.*, 2009). Food and drug administration (FDA) of U.S and Canadian Government in 2010 completely banned the export and sale of baby bottles that contained BPA (Food and Administration, 2010). Due to the high concentration of BPA being observed in the baby bottles, sippy cups and thermal recipient papers after being used American largest company of thermal recipient and thermal papers removed BPA formation (Raloff, 2010, Biedermann *et al.*, 2010). The restriction on the use of BPA resulted in the manufacturing of its alternatives into the market in the shape of bisphenol B [BPB; 2,2′-bis(4-hydroxyphenyl)butane] (BPB), bisphenol F [BPF; 4,4′-dihydroxydiphenylmethane] (BPF) and bisphenol S [BPS; 4,4′-sulfonyldiphenol] (BPS). All of the bisphenols (A, B, F and S) contain two phenol groups attached through carbon of other commercial structures (Rochester and Bolden, 2015, Rosenmai *et al.*, 2014, Chen *et al.*, 2016a). Since these all BPA analogues have structural similarity with BPA these all are expected to have same toxicological effects on the biological systems.

BPA exposure in human studies show its association with reduced ovarian response, implantation failure, miscarriage, reduced male sexual function, reduced sperm quality, altered sex hormone concentrations, altered liver function and oxidative stress in different organs

(Richter *et al.*, 2007, Bonefeld-Jørgensen *et al.*, 2007, Moriyama *et al.*, 2002, Vom Saal *et al.*, 2007). BPA has also been associated with abnormal gestation time, reduced birth weight, increase male genital abnormalities and obesity in children (Rubin *et al.*, 2001, HIROI *et al.*, 2004, Soto *et al.*, 2008). It has also been observed to alter behavior and neurodevelopment in children. The above all abnormities and complacencies have been supported by both *in vitro* and *in vivo* studies by different relevant human exposure doses experimentally (Midoro-Horiuti *et al.*, 2010, Miyawaki *et al.*, 2007, Toyama *et al.*, 2004, Berger and Shaw, 2008, Chitra *et al.*, 2003, Williams *et al.*, 2001). Several human studies also found association between maternal BPA exposure during gestation and neuroendocrine complacencies in the offspring (Braun *et al.*, 2011b, Cantonwine *et al.*, 2010, Chevrier *et al.*, 2013, Seminatti, 2017, Spanier *et al.*, 2012). BPA studies with postnatal BPA exposure also found endocrine alterations in the developing fetus (Bellinger *et al.*, 2007, Bellinger *et al.*, 2008, Maserejian *et al.*, 2012). In other studies it was found that populations with lower income have higher level of BPA in their blood and urine samples (Maserejian *et al.*, 2012, Melzer *et al.*, 2010, Carwile and Michels, 2011).

Introduction to BPA analogues bisphenol B (BPB), bisphenol F (BPF) and bisphenol S (BPS)

Bisphenol B (BPB)

BPB (2,2-bis (4-hydroxyphenyl) butane) is an analogue of BPA used for the production of phenolic resins and leaching dyes (Chen *et al.*, 2002b). BPB has also been found in the food items and beverages with similar leaking properties into food as BPA (Grumetto *et al.*, 2008). There have also been studies were BPB has been found in the endometriosis of women suggesting that it can also cross placental barriers (Cobellis *et al.*, 2009, Mendes, 2002). In the canned beverages BPB level has been found high in many tested samples (Cunha *et al.*, 2011, Cunha *et al.*, 2012, Cunha and Fernandes, 2013). BPB has also been detected in the sea food and indoor dust and also in the human blood, urine and umbilical cord samples (Liao *et al.*, 2012a, Liao *et al.*, 2012b, Lee *et al.*, 2015, Cunha and Fernandes, 2010). There have also been studies about the resistant in degradation of BPB as it is more resistant then BPA in both aerobic and anaerobic conditions (Chang *et al.*, 2014, Ike *et al.*, 2006).

Several studies have also shown that BPB has both estrogenic and anti-androgenic nature (Ike *et al.*, 2006, Kitamura *et al.*, 2005, Yoshihara *et al.*, 2001). BPB has also been observed to cause DNA damage and has been identified to be more potent agonist to human Pregnane-X receptor

than BPA showing much high toxicity potential than BPA (Rosenmai *et al.*, 2014, Pivnenko *et al.*, 2015, Sui *et al.*, 2012, Delfosse *et al.*, 2014, Chen *et al.*, 2002b).

Bisphenol F (BPF)

BPA another analogue BPF (bis (4-hydroxyphenyl) methane) contains two phenol rings similar to BPA but these rings are joined by methylene bridge. BPB has started gradually replacing BPA by having many applications in manufacturing industry of polycarbonates and epoxy resins used for the production of many household daily use items (Yamazaki *et al.*, 2015, Lee *et al.*, 2015, Yu *et al.*, 2015, Molina-Molina *et al.*, 2013). In the recent years there have been several studies which have detected BPF residues in food containers, epoxy resins and in water pumped by pipes made of BPF (Stroheker *et al.*, 2004, Goodson *et al.*, 2002, Usman and Ahmad, 2016). BPF has also been detected to a promising toxic concentration in the food stuff like fish, sea food, meat products, beverages and vegetables (Gallart-Ayala *et al.*, 2011a, Yamazaki *et al.*, 2015, Lee *et al.*, 2015, Rochester and Bolden, 2015). It has also been detected in the human urine samples and its presence has also been detected in the surface water, sediment samples and sewage water which indicates that it is turning to an environmental contaminant (Song *et al.*, 2014a, Ruan *et al.*, 2015, Yang *et al.*, 2014a, Lee *et al.*, 2015, Yamazaki *et al.*, 2015, Liao and Kannan, 2014a). Environmental protection Agency (EPA) of USA has confirmed that BPF is an endocrine disrupting chemicals (EDCs) which is hormonally as active as BPA. This was confirmed by a reported study which showed that BPF acts like an artificial glucocorticoid by interfering with the conversion of T3 and T4 levels in the serum (Zhang *et al.*, 2013, Rochester and Bolden, 2015, Kolšek *et al.*, 2015, Higashihara *et al.*, 2007). BPF has also been observed to induce oxidative stress in different tissues, induction in the lipid peroxidation and increasing ROS levels (Audebert *et al.*, 2012). It has also be found to have genotoxicity, endocrine toxicity and cytotoxicity (Pan *et al.*, 2014, Michałowicz *et al.*, 2015, Eladak *et al.*, 2015, Cabaton *et al.*, 2009).

Bisphenol S (BPS)

BPA family another member BPS (bis (4-hydroxyphenyl) sulfone) has two phenolic groups linked by sulphur dioxide group. In 1869 it was firstly introduced into the environment and later after few decades in 2006 it was used in the cash register receipts (Glausiusz, 2014). When BPA was banned so the companies switched to the so called safer analogues of BPA like BPB, BPF

and BPS and the use of BPS started gaining momentum in the production of baby bottles, thermal papers and epoxy resins (Becerra and Odermatt, 2012, Becerra and Odermatt, 2013, Grignard *et al.*, 2012, Liao *et al.*, 2012c, Rochester and Bolden, 2015, Liao *et al.*, 2012a, Rosenmai *et al.*, 2014). BPS is more photo and heat resistant and has been detected in several daily care products like food, indoor dust and water sources (Liao and Kannan, 2014a, Liao and Kannan, 2014b, Lee *et al.*, 2015, Rochester and Bolden, 2015, Rosenmai *et al.*, 2014, Pivnenko *et al.*, 2015, Michałowicz *et al.*, 2015). Due to high half-life and better dermal penetration of BPS it has been found in the general population in high concentration throughout the globe (Liao and Kannan, 2014a, Liao *et al.*, 2012d, Jackson, 2001, Liao *et al.*, 2012c). BPS is a non-degradable, non-environment friendly alternative to BPA and is becoming an ecological burden with every passing day (Danzl *et al.*, 2009, Yamazaki *et al.*, 2015, Song *et al.*, 2014a, Yang *et al.*, 2014a, Rochester and Bolden, 2015).

BPS widespread exposure among general population of various countries is becoming a problem and in the majority of urine samples collected from both Europe and Asian countries it was found that more than 80 % of the population had toxic concentrations of BPS on the daily basis (Chatrchyan *et al.*, 2012, Ye *et al.*, 2015, Yang *et al.*, 2014a, He *et al.*, 2009, Rochester and Bolden, 2015). BPS has toxic effects and in several studies it has been observed that it has hormonal potencies similar to BPA and it exerts acute cytotoxicity by inducing DNA damage (Chen *et al.*, 2002b, Ji *et al.*, 2013, Liao *et al.*, 2012c, Flint *et al.*, 2012). It has also been observed to affect the normal reproductive hormones in both male and female experimental animals (Ji *et al.*, 2013, Ullah *et al.*, 2017, Ullah *et al.*, 2018, Michałowicz *et al.*, 2015). BPS ability in inducing toxicity in many organ systems and it is thought that it may induce additional changes which we have not seen yet with BPA (Rosenmai *et al.*, 2014, Song *et al.*, 2014a, Chen *et al.*, 2016a).

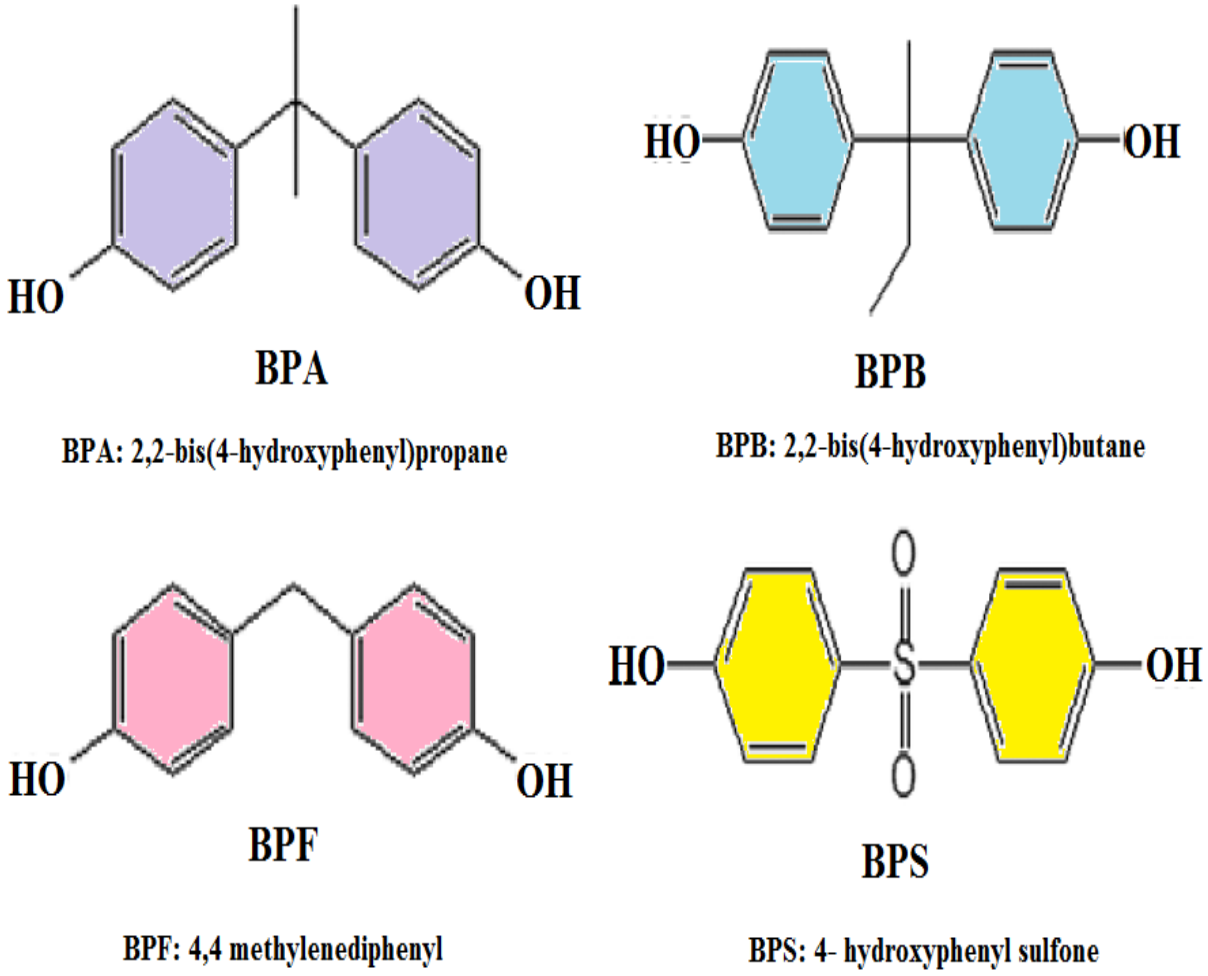

Fig 2. Chemical structures of bisphenol A (BPA) and its analogues bisphenol B (BPB), bisphenol F (BPF) and bispheonol S (BPS) used in the present study.

Toxicity of BPA analogues BPB, BPF and BPS

There have been several studies which have shown various toxic effects like endocrine disruption, cytotoxicity, genotoxicity, dioxin like effects and reproductive toxicity of BPA and its analogues BPB, BPF and BPS. Hormonal activities of BPA and its analogues showed the estrogenic, antiandrogenic, androgenic and antiestrogenic activities in several studies. BPA analogues have also shown similar potencies of that of estradiol which is critical for the cellular actions like differentiation, proliferation and apoptosis (Rogers *et al.*, 2013).

Estrogenic Effects of BPA and its Analogues BPB, BPF and BPS

In the development of reproductive organs and brain estrogens play an important role (Boon *et al.*, 2010, Hojo *et al.*, 2008, Bondesson *et al.*, 2015, Coumailleau *et al.*, 2015). BPA and its

analogues mimic and interfere with the actions of estrogen and act as endocrine disruptors (Vandenberg *et al.*, 2012). Environmental exposure to BPA and its analogues is associated with a wide range of toxic effects in humans, rodents and wildlife (Rochester, 2013). BPA analogues are also related to numerous adverse health effects like diabetes, obesity, behavioral problems and reproductive disorders (Rezg *et al.*, 2014). In the cell culture and binding assays BPS and BPAF have been observed to bind to estrogen receptors and exert estrogenic activities at the transcriptional level (Matsushima *et al.*, 2010, Grignard *et al.*, 2012, Ullah *et al.*, 2016). There have also been studies that BPA and its analogues BPB, BPF and BPS have potential role in interfering and disrupting the normal functions of endocrine system (Ullah *et al.*, 2016, Rochester and Bolden, 2015, Naderi *et al.*, 2014). There is very limited data available about BPA and its analogues BPB, BPF and BPS impact on the brain development and on the endocrine system in mammals (Negri-Cesi, 2015, Castro *et al.*, 2015, Kinch *et al.*, 2015).

Neuroendocrine disruption due to exposure of BPA analogues BPB, BPF and BPS

Neurobehavioral changes of BPA analogues show increased velocity, increased anxiety, reduced motivation in social interactions, decreased body weight, increased anxiogenic behavior and depressive state in dams exposed to 10-50 mg/kg/body weight (Rosenmai *et al.*, 2014, Kim *et al.*, 2015, Ohtani *et al.*, 2017). In another study exposure to BPA and its analogues compromised the maternal care and exhibited increased expression in the prefrontal cortex in response to corticosteroid synthesis (Castro *et al.*, 2015). Analogues of BPA induce neuroendocrinological changes in rodents, a study by Vinas and Watson in 2013 showed that BPS disrupted membrane initiated cell signaling, resulting in cell death and in another study by Castro and collogues showed that BPA and its analogues affect differently 5α-reductase expression and dopamine-

serotonin systems in the prefrontal cortex (Castro *et al.*, 2015, Viñas and Watson, 2013).

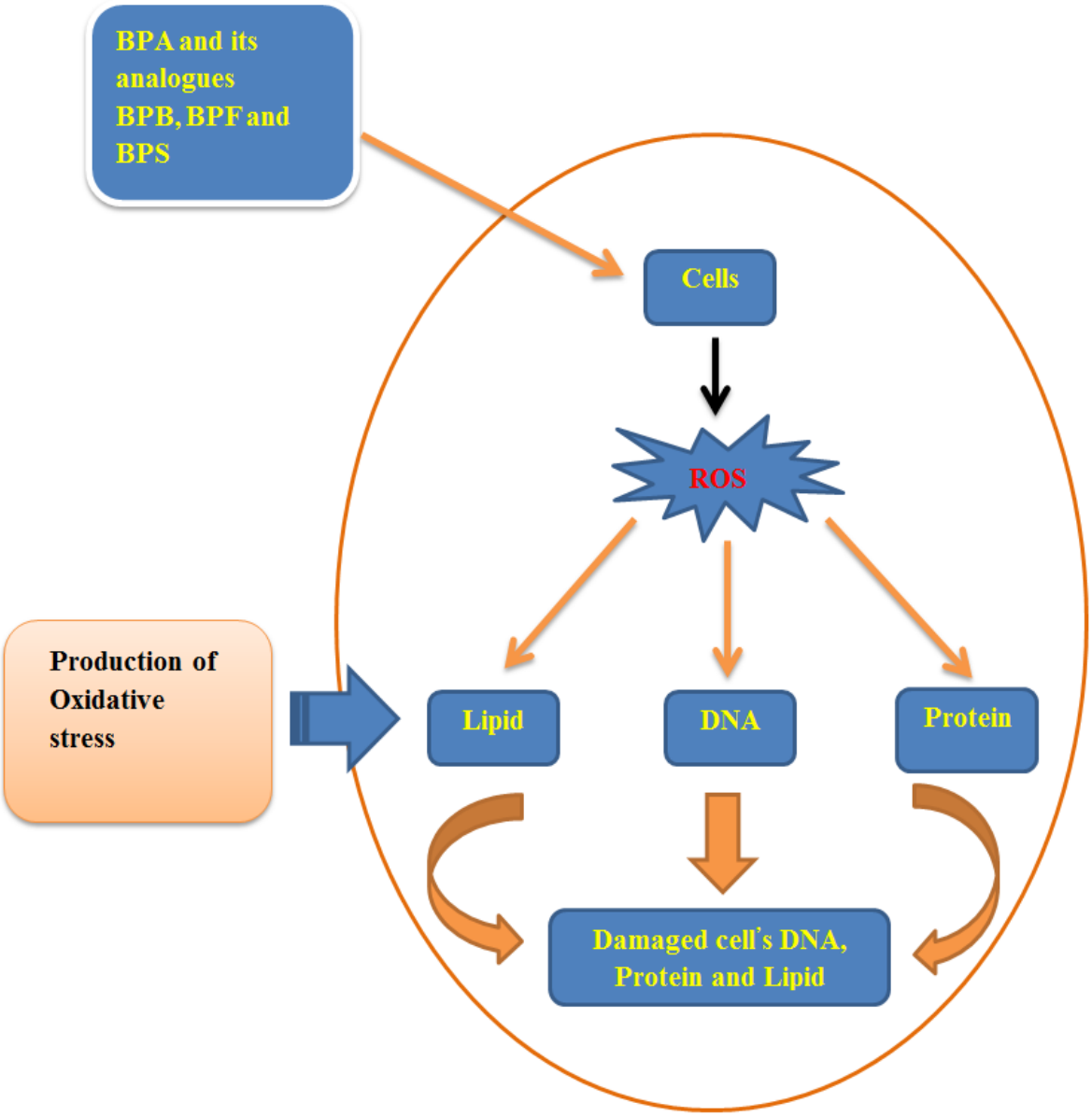

Fig 3. Mechanism of action of bisphenol A and its analogues BPB, BPF and BPS through production of oxidative stress in the body.

Endocrine Disruption of BPA analogues BPB, BPF and BPS

In the past few years the estrogenic and anti-androgenic potencies of BPA analogues have been under intensive investigation. BPA analogues have shown similar or greater estrogenic potencies when comparative toxic effects of bisphenols were checked (Kang *et al.*, 2014, Stossi *et al.*, 2014, Rosenmai *et al.*, 2014, Stroheker *et al.*, 2004, Molina-Molina *et al.*, 2013, Rochester and

Bolden, 2015, Matsushima *et al.*, 2010). Some of the BPA analogues tested so far have shown higher estrogenic potencies than BPA (Kang *et al.*, 2014, Kitamura *et al.*, 2005). Antiandrogenic effect of BPA analogues have been studied on the inhibitory concentration for half maximal competitive binding receptor (Rosenmai *et al.*, 2014, Molina-Molina *et al.*, 2013, Roelofs *et al.*, 2015). In human breast cancer luciferase reporter gene assay test it has been observed that several analogue of BPA show high estrogenic activity or similar to BPA (Kitamura *et al.*, 2005). Another study showed that analogues of BPA have effects on the estrogen and androgen receptor and these analogues show potencies with in the same order of magnitude as compared to BPA (Rosenmai *et al.*, 2014, Kitamura *et al.*, 2005). Though in some studies it has been observed that BPS is less estrogenic and have less antiandrogenic activities than BPA (Rosenmai *et al.*, 2014). In another study in hER assay it was observed that BPA, BPF and BPS activated both receptor of estrogen (hERα and hERβ) but BPS was more active in hERα while showing higher values for androgen receptor (hAR) (Molina-Molina *et al.*, 2013). A study by Eladak et al in 2015 showed that BPS, BPF and BPA decreases basal testosterone secretion in humans showing antiandrogenic effects of these BPA analogues (Eladak *et al.*, 2015). In another steriodogensis assay of H295R Goldinger et al 2015 showed that BPA and BPF induce low production of 17β-estradiol and free production of testosterone (Goldinger *et al.*, 2015). Stossi et al also found the antiandrogenic, antiesterogenic activities of BPA analogues by multiparametric microscopy platforms (Stossi *et al.*, 2014). In this study it was also observed that these analogues either act as agonist, mixed agonist or antagonist for the ERα and ERβ receptor. In another study it was found that BPF has antiestrogenic effects for 17β-estradiol whereas BPS act as a weak agonist for human endocrine receptor (Stroheker *et al.*, 2004). In another study by Matsushima et al in 2010 it was observed that BPAF is a good agonist of ERα and a strong antagonist for ERβ (Matsushima *et al.*, 2010). Where it acts as a very strong antagonist for the activity of 17β-estradiol and its potency was much higher than BPA.

Table 1. Calculated physiochemical properties of bisphenol A and its analogues BPB, BPF and BPS

BCF = bioconcentration factor (L/Kg wet weight)

BAF = bioaccumulation factor (L/Kg wet weight)

Bisphenols	CAS	MW (g/mol)	Half-life (day)				BCF	BAF
			Atmosphere	Water	Soil	Sediment		
BPA	080-05-7	228.29	0.067	37.5	75	337.5	172.7	172.8
BPB	77-40-7	242.31	0.066	37.5	75	337.5	170.2	170.3
BPF	620-92-8	200.23	0.065	15.01	30	135.0	28.02	28.02
BPS	080-09-1	250.27	0.368	15.01	30	135.0-	3.535	3.535

Table 2. Toxic effects of bisphenol A and its analogues as BPB, BPF and BPS

	BPA	BPB	BPF	BPS
Estrogenic	±	±	±	±
Androgenic	±		±	±
Anti-estrogenic	±	±	±	±
Anti-androgenic	±		±	±
Cytotoxicity	±		±	
Genotoxicity	±	±	±	±
Reproductive toxicity	±			
Neurotoxicity	±		±	
Acute toxicity	±			±

The ± symbol represent the known effects of the chemical so far.

Cytotoxicity and genotoxicity of BPA analogues BPB, BPF and BPS

Several studies have shown that BPA analogues have cytotoxicity and genotoxicity and in some conditions these BPA analogues have even shown stronger genotoxic potencies than BPA. Due to the lack of studies on these BPA analogues we still lack endocrine studies which can show the most effected doses for both the genotoxic and cytotoxic effects on these endocrine disruptors (Liao and Kannan, 2014b). A study by Audebert *et al* 2011 on human hepatoma HepG2 cell lines showed the cytotoxicity for BPA and BPF where only genotoxicity was observed for BPF (Audebert *et al.*, 2011). Human peripheral blood mononuclear cells treated with BPA, BPF and BPAF decreased it viability (Audebert *et al.*, 2011). In another study by Cabaton et al 2009 showed that HepG2 cells after 24 h exposure with BPA analogues as BPB, BPF, BPZ and BPAF showed DNA fragmentation at non cytotoxic concentrations by inducing significant DNA damage in those cells (Cabaton *et al.*, 2009). In another comparison study with BPA analogues BPF and BPAF it was observed that all the bisphenols enhanced the formation of reactive oxygen species (ROS) (Michałowicz *et al.*, 2015). A study on genome wide gene expression of BPA and its analogues BPS and BPAF exposure lead to estrogen dependent osteosarcoma in cells with each of these chemicals exposure and certain significant effects of gene expression were also evident after exposure with these chemicals (Fic *et al.*, 2013a). Among the significant effects of gene expression it was observed that all the tested bisphenols affected the gene expression related to fetal development (Fic *et al.*, 2015). Another study on mutant chicken DT40 cells showed that BPAP, BPP and BPM have higher genotoxic potentials than BPA (Lee *et al.*, 2013). BPA analogues BPS and BPF affected differently the 5α-reductase expression and dopamine (DA) serotonin (5-HT) systems in the prefrontal cortex of female juvenile rats in an *in vivo* study by (Castro *et al.*, 2015) where it was also observed that BPF and BPS decrease 5α-R3 mRNA levels and BPA decrease 5α-R2 and 5α-R3 mRNA at the protein levels (Bisphenol, 2015).

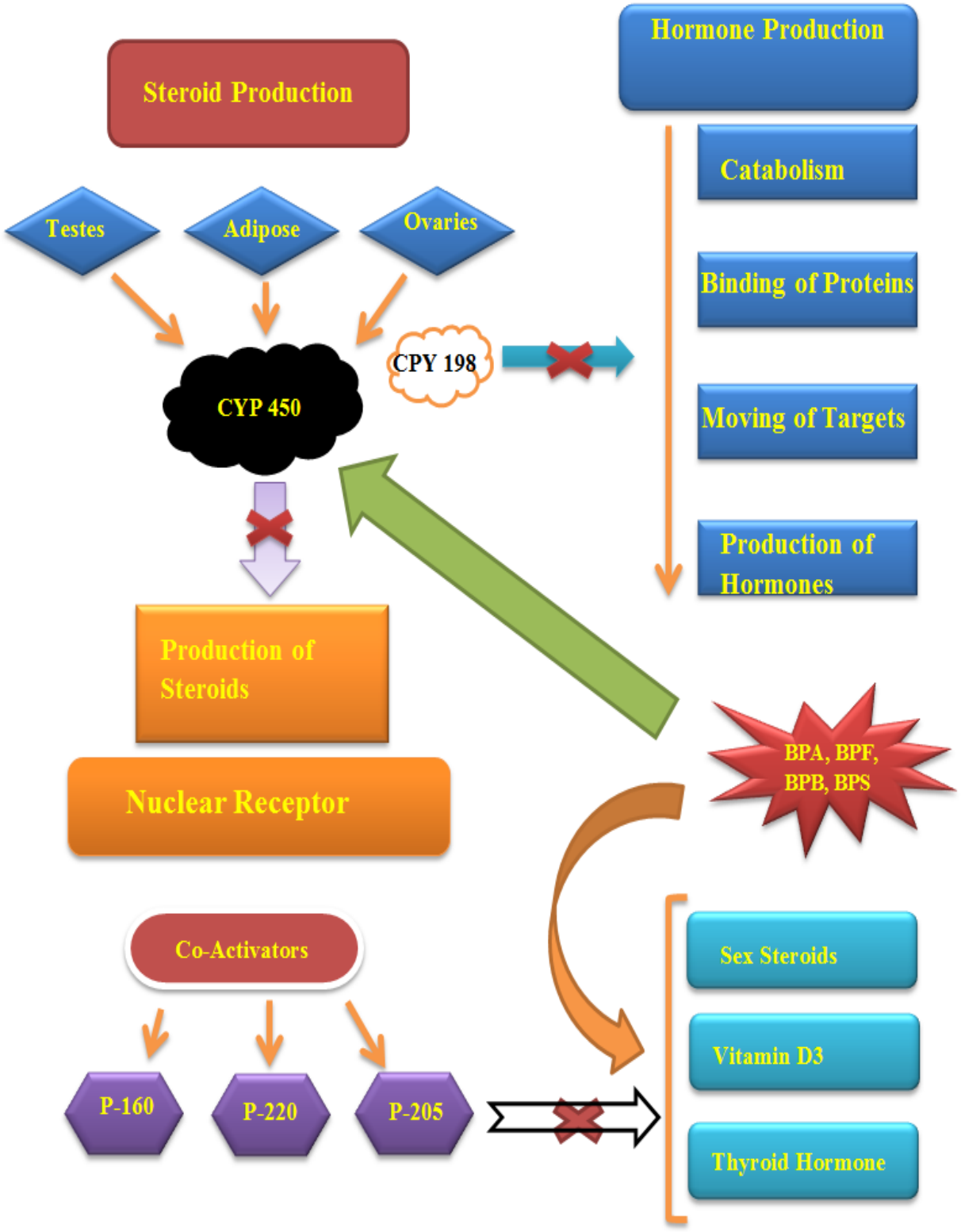

Fig 4. Mechanism of action of bisphenol A and its analogues BPB, BPF and BPS at different hormonal and receptor level.

Reproductive neurotoxic effects of BPA analogues BPB, BPF and BPS

BPA and its analogues BPB, BPF and BPS have been observed to have toxic effects, including cytotoxicity, genotoxicity and endocrine disruption. Studies have shown very clearly that BPA analogues like BPB, BPF and BPS have potency similar to BPA for estrogenic, antiestrogenic, androgenic and antiandrogenic receptors (Rochester and Bolden, 2015). In a study on zebrafish BPS exposure significantly reduced the number of eggs and altered the gonadosomatic index (Ji *et al.*, 2013). When F1 zabrfish embryos were exposed to BPS different concentrations it was observed that the hatchability decreased and malformation increased. Exposure to BPS also disturbed the normal development and caused disturbance in the feedback regulatory index at the hypothalamus pituitary gonadal (HPG) axis. Studies have also shown that hormonal balance and reproductive potentials are also impaired after exposure to BPS at the developmental level (Ji *et al.*, 2013, Naderi *et al.*, 2014). In another study when zebrafish embryos were exposed to BPA and BPS it was observed that neurogenesis increased at the level of hypothalamus and also resulted in the hyperactive behavior in the later stages (Kinch *et al.*, 2015). In another study exposure to BPA and BPS treatment to mouse 3T3-L1 adipocytes increased glucose uptake and leptin production (Héliès-Toussaint *et al.*, 2014). These all findings at this stage suggest that both BPA and BPS are involved in the obesity, metabolic pathways and different reproductive mechanisms (Ma *et al.*, 2015). BPA analogues are also involved in the toxicities similar to BPA and these all analogues poses similar mode of actions raising the safety concerns on the applications of BPA replacements (Chen *et al.*, 2002b, Yokota *et al.*, 2008, Ullah *et al.*, 2018). BPA analogues toxicity studies remain remarkably limited so far regarding mode of actions and quantitative toxicity in both *in vivo* and *in vitro* experimental models.

Effects of metabolic modifications on toxicity of BPA and its analogues BPB, BPF and BPS

Studies on the laboratory animals in the past have shown that both metabolic and excretion pathways of BPA analogues BPB, BPF and BPS are not very different of BPA (Liao and Kannan, 2012). Studies in the past have shown both free and conjugated BPA and its analogues in the human urine samples (Liao *et al.*, 2012c, Liao *et al.*, 2012a, Liao and Kannan, 2011, Zhang *et al.*, 2011b, Liao *et al.*, 2012d). Another study by Okuda et al 2011 showed the metabolites of different analogues of BPA after incubation with rat live fractions (Okuda *et al.*, 2011). BPF has also been observed to be involved in the metabolic toxicity after female rats were exposed to its different concentrations (Kitamura *et al.*, 2005). In another study BPF

residues were also found in urine and feces and the elimination rate of BPF was also lower than BPA (Cabaton *et al.*, 2006). BPA analogues as BPAF, BPC, BPF and BPZ were observed in the presence of triphosphopyridine nucleotide (NADPH) with hydroxylated metabolites conjugates by largely increasing the toxicity of these bisphenols (Schmidt *et al.*, 2013). BPA has also been found in conjugated form with estrone and sulfate in the growth of breast tumor cells (Boucher *et al.*, 2015). Therefore sulfation may increase the estrogen potential of BPA (Stowell *et al.*, 2006, Yoshihara *et al.*, 2004, Kitamura *et al.*, 2005). *In vitro* assays in rat liver S9 cells revealed that BPS metabolite in the liver showed elevated estrogenic activity (Schmidt *et al.*, 2013). *In vivo* study has also revealed that BPF showed elevated cytotoxic effects in rats after exposure to its different concentrations (Audebert *et al.*, 2011). Overall, the limited number of metabolism studies has indicated the effects of metabolic modifications on the toxicities of BPA analogues.

Sources and routes of exposure of BPA and its analogues BPB, BPF and BPS

BPA and its analogues BPB, BPF and BPS have also been documented in the environmental compartments such as drinking water, wastewater, waste water plant and distribution pipes, sediment, sewage, food and beverages (Ballesteros *et al.*, 2006, Bulloch *et al.*, 2015, Gallart-Ayala *et al.*, 2007, Kosaka *et al.*, 2012, Fan *et al.*, 2013, Lane *et al.*, 2015, Song *et al.*, 2014b, Song *et al.*, 2014a, Bourgin *et al.*, 2013, Zhou *et al.*, 2014). A better approach for the identification and quantification of BPA and its analogues BPB, BPF and BPS in drinking water reaching house hold unites to estimate human exposure is yet to be understood. Chlorinated items used for the household cleaners like dishwashing, toilet cleaner and laundry detergents and personal care products could be a main source of BPA and its analogues exposure to human (Dodson *et al.*, 2012, Odabasi, 2008, Leri and Anthony, 2013). Several studies in the past have shown that BPA analogues were also measured in the fatty tissues and compared to the corresponding urine ratios in daily exposure levels (Leri and Anthony, 2013, Jimenez-Diaz *et al.*, 2010, Migeot *et al.*, 2013).

Toxicity and Health Outcomes of BPA and its analogues BPB, BPF and BPS from *in-vitro* and *in-vivo* animals to human studies

BPA and its analogues BPB, BPF and BPS have been studied extensively in both *in vitro* and *in vivo* studies and the human health studies of BPA analogues still are very limited (Migeot *et al.*, 2013, Rochester and Bolden, 2015, Rosenmai *et al.*, 2014). There are some studies which have shown that BPA analogues alter metabolism and induce oxidative stress which give clear

indication of BPA and some of its analogues alterations at the cellular oxidants (Babu *et al.*, 2013, Kitamura *et al.*, 2005).

Comparative analysis of the undesirable effects of BPA and its analogues BPB, BPF and BPS

Several studies in the past have shown the presence of BPA and its analogues BPB, BPF and BPS in the environment have started to increase the contamination. BPA analogues transport into the biological system and the presence of BPA analogues in the human bio specimen have started to increase with every passing day (Andra *et al.*, 2015). Many of these analogues have also shown that BPA like hormonal activities and that these analogues are increasing the release of endocrine disrupting chemicals in the environment around us (Rochester and Bolden, 2015, Moriyama *et al.*, 2002). BPA analogue like BPS, BPB and BPF are likely to accumulate in the environment due to resistant in the degradation. Another study showed that BPF is turning to become a major problem due to slow metabolized nature (Stroheker *et al.*, 2003). Some of the BPA analogues are also found to be toxic and possess strong genotoxic natures which induce oxidative stress in many animal model studies (Liao *et al.*, 2012c, Fic *et al.*, 2013a, Eladak *et al.*, 2015). There are even studies which have shown that BPA analogues BPB, BPF and BPS tend to be more potent then BPA itself (Okada *et al.*, 2008, Zhuang *et al.*, 2014, Nakagawa *et al.*, 2007). However, epidemiological and experimental studies of these analogues analyzing the endocrine disruption and toxicity still need to be undertaken to analyze harmful effects of these analogues on the different body systems.

Effects of metabolic modifications of BPA and its analogues BPB, BPF and BPS

BPA and its analogues BPB, BPF and BPS toxicity is largely affected by natural metabolism. Bisphenols (BPA, BPB, BPF and BPS) excretion by urine is mainly facilitated by conjugation of bisphenols with β-glucuronide and sulfate which is the main metabolic pathway. It has been noted that BPA and its analogues BPB, BPF and BPS induce adipocytes differentiation in human (Kitamura *et al.*, 2005, Okuda *et al.*, 2011). Overall, the limited number of metabolism studies have indicated the effects of metabolic modification on the toxicities of BPA analogues (Okuda *et al.*, 2011). Elucidation of metabolic pathways and products should be emphasized during risk assessments of BPA analogues (Boucher *et al.*, 2015). BPA analogues conjugates were subjected to deconjugation in studies with newborns (Stowell *et al.*, 2006). This deconjugation in newborn was done by arylsulfatase C which is mainly developed in the early stages of life (Ginsberg and

Rice, 2009). The above studies show that if rapid metabolism is ensured then the rest associated to BPA analogues toxicity can be limited (Nahar *et al.*, 2013, Nakamura *et al.*, 2011). BPA and some of its analogues incubated with liver S9 fraction cells were found to produce dimerized metabolites via multiple pathways (Nahar *et al.*, 2015). These metabolites produced microsomes and cytochrome P450 with an addition of NADPH and GSH (Okuda *et al.*, 2011). The limited number of metabolic studies have indicated that the effects of metabolic modifications on the reproductive toxicity of BPA analogues are still to show the pathways and products during risk assessment of BPA analogues (Audebert *et al.*, 2011).

Due to widespread exposure of BPA and its analogues BPB, BPF and BPS which lead to many metabolic and reproductive disorders have imposed restrictions on the use of BPA which has in return led to a shift in the use of its analogues like BPB, BPF and BPS etc. The production of these BPA analogues has increased in the past and it is at big rise in the future. As a result, BPA analogues have already shown their presence in various environmental compartments as well as food and beverages by increasing the risk of occupational and general population exposure. These analogues also act as BPA due to their structural similarity and these similarities also make them common endocrine disruptors.

AIMS AND OBJECTIVES

The main objectives of the current study were to investigate the comparative toxicological and endocrine disrupting potentials of BPA and its analogues BPB, BPF and BPS in male rats as experimental models through both *in vitro* and *in vivo* studies. There are several studies in the past which have shown the toxic effects of BPA by altering the endocrine function but there is very limited data available showing toxic and endocrine disrupting effects of BPA its analogues BPB, BPF and BPS. Studies on BPA and its analogues as BPB, BPF and BPS exposure alter endocrine hormones functions by inducing cellular toxicity (Delfosse et al., 2014; Kinch et al., 2015; Liao & Kannan, 2014b; Liao, Liu, Guo, et al., 2012). Hormones play important role in the developmental stages of life and at this period if animals are exposed to EDCs they imparts serious negative effects on the development and sexual differentiation of an animal. Exposure to EDCs at the gestational and neonatal period leads into permanent alterations in the mechanism of action of hormones which can cause disturbances to developmentally related structures. There is very limited data available regarding developmental toxicity of BPA and its different analogues showing endocrine disrupting potentials of these analogues on the hypothalamic pituitary testicular axis (HPT axis). BPA and its analogues BPB, BPF and BPS oxidative stress inducing potentials and its effects on the endocrine functions in the reproductive system remain unknown.

1. Objectives of the first study (*in vitro* studies)

Our first objective was to understand the comparative hazardous effects of BPA and its analogues BPB, BPF and BPS on spermatogenesis and sperm DNA integrity through oxidative stress by using *in vitro* approaches.

Conclusions

In the present study, antioxidant enzymes status of the testicular tissues was depleted and oxidative stress was induced in the reproductive tissues after exposure to BPA and its analogues BPB, BPF and BPS. The present *in vitro* study results suggest that BPA and its analogues BPB, BPF and BPS exposure changed antioxidant enzymes activity and caused sperm DNA damage in the rat testis which suggest the relation of sperm motility and DNA damage.

2. Objectives of the second study (*in vivo* sub-chronic studies)

In the second experiment, comparative sub-chronic reproductive toxicity of BPA and its analogues BPB, BPF and BPS was determined in the reproductive system of male rats and we also observed the oxidative stress inducing potentials of BPA and its analogues in the reproductive tissues of male rats.

Conclusions

Findings of our present investigations suggest that BPA and its analogues BPB, BPF and BPS not only show anti-androgenic properties but also lead into oxidative stress which causes disturbances in the reproductive function of adults rats. The present comparative studies on BPA and its analogues BPB, BPF and BPS also suggest the toxic effects of BPA and its analogues BPB, BPF and BPS on testis and spermatogenesis.

3. Objective of the third study (chronic study)

In the third study, we tried to investigate the low concentrations chronic reproductive toxicity induced by BPA and its analogues BPB, BPF and BPS in the male reproductive system.

Conclusions

On the basis of the results from the present study, it can be concluded that chronic exposure for a long period of time to low concentrations of BPA and its analogues BPB, BPF and BPS are capable of suppressing gonadotropins secretion from pituitary, exhibiting estrogenic and anti-androgenic effects in the mammals, inducing oxidative stress in the testicular tissue and affecting spermatogenesis by causing maturational arrest at spermatogoneal stage as well as at the stage when spermatids can be seen.

4. Objectives of the fourth study (pre-natal study)

The present study was designed to understand the estrogenic mode of actions and toxicity inducing potentials of BPA and its analogues BPB, BPF and BPS on sexual development of pre-natal male rats.

Conclusions

The results of the present study on BPA and its analogues BPB, BPF and BPS showed toxic effects on the sexual development recognizing that BPA and BPB, BPF and BPS exposure to

mother during pregnancy may induce reproductive toxicity in the offspring. Low concentrations of BPA and its analogues can have effects on the organs and sexual development of adult rats.

5. Objectives of the fifth study (post-natal study)

Present study aims to investigate possible toxic effects of BPA and its analogues BPB, BPF and BPS on testicular development in rats exposed during neonatal stage of life. Although the toxicity of BPA has been studied in detail but such information on its analogues is still scarce.

Conclusions

In the present study we observed that exposure to different concentrations of BPA and its analogues BPB, BPF and BPS during neonatal period bring about prominent changes in the endocrine system of male rats by altering hormonal profile and affecting sperm parameters. In the present study we also observed reduction in the viability and motility of sperms and arrest of spermatogoneal cells after neonatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS.

ABSTRACT

Background: Bisphenol A (BPA) has a very long story in the history of science used as main component of many consumer products like infant's feeding bottles, coatings of beverages and food cans. BPA can spread into environment and has been detected in saliva, blood and food. BPA leakage into many consumer products led ban in many countries where alternatives to BPA were introduced into market. BPA alternatives such as bisphenol B (BPB), bisphenol F (BPF) and bisphenol S (BPS) have similar chemical structure and binding ability for estrogen receptor (ER) as BPA and have shown toxicological effects in animal models.

Materials and methods: In the present study, comparative effects of exposure to BPA and its analogues BPB, BPF and BPS on oxidative stress, antioxidant activity and testosterone concentrations in the rat testis were evaluated by *in vitro* approaches. Testes and sperms were incubated with different concentrations of BPA and its analogues BPB, BPF and BPS (0, 1, 10, and 100 ug/L). After two hours of incubation, antioxidant enzymes concentrations, oxidative stress markers and testosterone concentration were estimated and analyzed.

Results: BPA and its analogues BPB, BPF and BPS exposure involved in decreasing antioxidant enzymes activity and induced oxidative stress. Higher concentrations of BPA and its analogues caused increase in lipid peroxidation, DNA fragmentation and production of reactive oxygen species (ROS) in the spermatozoa of rats. These chemical were capable of altering steroidogenesis hence led to the decrease in testosterone secretions.

Conclusions: The present comparative study on BPA and its analogues BPB, BPF and BPS suggest the toxic effects of these chemicals on testes induce oxidative stress and alter the process of spermatogenesis.

INTRODUCTION

Bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl) propane) is one of the high produced chemicals in the world, has been used for the production of many plastic consumer products like food containers, water pipes, paper products, electronics, toys and medical equipments (Vandenberg *et al.*, 2009). Human and other animal are exposed to BPA by both dietary and non-dietary sources (Vandenberg *et al.*, 2007, Geens *et al.*, 2012a). BPA presence in the human urine samples, breast milk, umbilical cord and placental tissues has been reported in many studies previously has revealed its worldwide exposure (Rochester, 2013). In both *in vivo* and *in vitro* studies, BPA effects on development, reproduction, metabolism, cardiovascular disease and neuronal networks has been well documented (Richter *et al.*, 2007, Bonefeld-Jørgensen *et al.*, 2007). Worldwide exposure to BPA has lead regulations on its production and in 2010 its use in baby bottles was banned in Canada and European union (EU) (Vom Saal *et al.*, 2007, Crain *et al.*, 2007, Chen *et al.*, 2016a). Ban on BPA use in many applications has lead into the production of many alternative substances which are structurally similar to BPA and are currently used for the production of epoxy resin and polycarbonate plastics (Rosenmai *et al.*, 2014). These chemicals are known as BPA analogues because of their common structure of two hydroxyl phenyl functionalities. BPB, BPF and BPS are among the main substitutes of BPA having broad range of applications such as used in epoxy glues, thermal paper receipts, coatings for food/beverages packaging, varnishes, dental sealants, adhesives, water pipes, electronics, polyesters, dyes, tanning agents, oral prosthetic devices, plastic optical fibers and wave guides. The use of these BPA analogues is at high rise globally (Rosenmai *et al.*, 2014, Cabaton *et al.*, 2009, Naderi *et al.*, 2014, Matsushima *et al.*, 2010, Chen *et al.*, 2016a).

Although, in comparison to BPA itself, studies on the BPA analogues are very limited in number. BPA some analogues are likely to have toxic effects including cytotoxicity, reproductive toxicity, neurotoxicity and endocrine disruption (Chen *et al.*, 2016a, Choi *et al.*, 2004, Masuo and Ishido, 2011, Meeker *et al.*, 2009b, Ullah *et al.*, 2018). A study on BPA analogues showed that BPS and BPF have similar potency for androgenic, antiandrogeic, estrogenic and antiestrogen activities (Rochester and Bolden, 2015). Some reports have also shown that BPA analogues interfere and disrupt the endocrine function and lead into endocrine disrupting activities in organisms both *in vivo* and *in vitro* studies (Feng *et al.*, 2012, Le Fol *et al.*, 2017, Yang *et al.*, 2014a, Eladak *et al.*, 2015, Cano-Nicolau *et al.*, 2016a, León-Olea *et al.*,

2014, Negri-Cesi, 2015, Castro *et al.*, 2015). Recently, a few studies showed that in addition to BPA, there has been an increase in the concentration of other BPA analogues as BPB, BPF and BPS in many of the beverages and food products across the united states and in several Asian countries (Liao and Kannan, 2013, Liao *et al.*, 2012b). There are studies which have shown that BPA has negative impact on the neuronal development and BPA analogs can also interfere the normal functions of endocrine system in several organisms (Cano-Nicolau *et al.*, 2016a, Liao *et al.*, 2012b, Molina-Molina *et al.*, 2013).

While comprehensive data is available about BPA toxic effects on both human and other animal models, these toxic effects of BPA analogues are still to be investigated. BPA alternatives are very similar to BPA and it is expected that these may have similar toxicological properties. Endocrine disrupting potentials of some these BPA analogues is only known so far where it is expected that these alternatives may possess harmful and genotoxic effects (Rochester and Bolden, 2015). Studies have also shown the genotoxicity, oxidative stress and DNA damage of BPA analogues in many animal models (Rochester and Bolden, 2015, Kolšek *et al.*, 2012, Hu *et al.*, 2012). BPA analogues BPB, BPF and BPS resulted in oxidative stress in testes and altered reproductive function in rats (Ullah *et al.*, 2016, Ullah *et al.*, 2018). These analogues have also been found in the foodstuff and human blood and urine samples across the united states (Liao and Kannan, 2013).

On the basis of the our previous study (Ullah *et al.*, 2018) indicating oxidative stress inducing potentials of BPS, the present study was aimed to investigate the comparative hazardous effects of BPA and its analogues BPB, BPF and BPS on spermatogenesis, sperm DNA integrity through oxidative stress by using *in vitro* approaches. The result of the present findings will be beneficial in understanding the toxic potentials and health hazards of BPA and its substitutes for the future industrial applications.

MATERIALS AND METHODS

Chemicals and experimental animals

BPA, BPB, BPF and BPS 99% purity were purchased from Santa Cruz biotechnologies, USA. For the *in vitro* study media containing Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were all purchased from thermos Fisher Scientific (Waltham, MA, USA). H_2O_2, Ca^{2+}, Mg^{2+}, Hank's balance salt solution (HBSS) and CAT, N-acetyl-L-cysteine (NAC) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sprague Dawley adult male rats (age 80 to 90 days) were obtained from the Primate/rodent Facility of Department of Animals Sciences, Quaid-i-Azam University, Islamabad, Pakistan. Animals were housed in steel cages and each cage contained a maximum of 5 animals. Prior to the start of the experiment standard laboratory conditions were maintained and animals handling ethical committee guide lines of the Department of Animals Sciences were followed. Room temperature was maintained at 22-25 ℃ and light/dark cycle was maintained for all the animals throughout the experimental period. Animals were fed with laboratory feed and tap water was available freely for the animals. Protocols of handling of the animals were approved by the Ethical committee of Quaid-i-Azam University, Islamabad, Pakistan.

Experimental design for the *In vitro* experiment

Sprague Dawley male adults rats (n=13) were used in this study. *In vitro* experiment was performed to investigate the effects of direct exposure of BPA and its analogues BPB, BPF, BPS on the testosterone production and testicular antioxidant enzymes status. Different doses of BPA and its analogues (0, 1, 10 and 100 µg/L) were used. The doses were selected as described earlier by (Hulak *et al.*, 2013, Ullah *et al.*, 2016). Stock solutions of bisphenols (BPA, BPB, BPF and BPS) were prepared in ethanol. *In vitro* culturing of sperms and testicular tissues was done according to the protocol described by (Ullah *et al.*, 2016) with slight modifications.

Testis slices preparation and incubation

Testicular tissues were dissected from the euthanized animals and washed with cold buffer saline. The dissected testes were cut in five equal parts and were processed in tubes. Two milliliter (mL) of Dulbecco`s modified eagle`s medium/Ham F12 (DMEM/Ham F12 mixture medium 1:1 ratio, Gibco, USA) containing 1.2 g/L sodium bicarbonate and supplemented with 50 IU/mL penicillin and 50 µg/mL streptomycin were mixed with 0, 1, 10, 100 ng/ml of BPA

and its analogues BPB, BPS and BPF and culture tubes were incubated for two hours in CO_2 incubator. Tubes were placed in pre-heated CO_2 incubator at 33 ℃ with 5% CO_2 and 95% air. After two hours of incubation the tissues were removed from the culture media and washed with saline. The cultured tissues 90 mg was homogenized in 3 ml of Phosphate buffer saline and centrifuged at 30,000 for 30 min. The supernatant of homogenate was collected and stored at -80 °C for hormonal assay and antioxidant.

***In Vitro* sperm preparation and incubation**

As the rats were euthanized epididymis was removed and washed in cold buffer saline. The cauda part of the epididymis was cut and crushed in buffer solution containing (NaCl, EDTA, glycerol and tris base) with maintaining the pH of the solution up to 7. The samples were mixed and later centrifuged for 10 mins at 3000 rpm. After the centrifugation the solution supernatant was discarded and sperms were taken from the solution and further diluted with media containing (Ham's F 12 media, Sigma Aldrich, Germany) and serum up to $1x10^8$ of the sperm per mL. The sperm samples were further processed with different concentrations (0, 1, 10, 100 µg/L) of BPA and its analogues BPB, BPF and BPS in carbon dioxide incubator with the temperature of 33 °C for the next two hours. After the two hour incubation period all the samples were again centrifuged at 1000 rpm for further 10 minutes. The centrifuged samples supernatant was discarded and the sperm pellet samples were further divided in two equal parts for the determination of antioxidant enzymes assays, oxidative stress markers and other part was used for the determination of comet assay. All the samples were stored in -80 °C freezers until further biochemical parameters.

Biochemical analysis and antioxidant enzymes

Male rats reproductive tissues (Testis) were homogenized and supernatant of the testis was further used for the different antioxidant enzymes and oxidative stress markers. The same way the sperm homogenate sample was also processed for the above two biochemical parameters.

Catalase (CAT)

CAT activity was determined by the method used by (Aebi, 1984). Tissue homogenate (50 µl) was diluted with 2 ml of 50 mM phosphate buffer (pH 7.0). Two ml of diluted homogenate was added with 1ml of 50 mM phosphate buffer (pH 7.0) containing 30 Mm H_2O_2 to sample tube, similarly distilled water was added to blank samples instead of homogenate. The above all

samples were mixed immediately and the absorbance was read after 15 seconds and 30 seconds at 240 nm. One unit of CAT activity was defined an absorbance change of 0.01 as U/min.

Superoxide dismutase (SOD)

SOD activity was determined according to the method developed by (Kakkar *et al.*, 1984). For the determination the determination of SOD 0.3 mL homogenate, 0.1 mL of phenazine methosulphate (186 μmol/L) and 1.2 mL of sodium pyrophosphate buffer (52 μmol.L, pH, 7.0) were added. The reaction was initiated by adding 0.2 mL of NADH (780 umol/L) and stoped by adding 1 mL of glacial acetic acid. The amount of chromogen formed was measured by recording the absorbance at 560 nm using a spectrophotometer. The results were expressed in U/mg protein and mU/108 sperm.

Peroxidase (POD)

POD activity in homogenate was determined by spectrophotometric method of (Carlberg and Mannervik, 1975). The reaction solution contained 0.1 ml homogenate, 0.1 ml of 20 Mm guaiacol, 0.3 ml of 40 mM H_2O_2 and 2.5 ml phosphate buffer 50 mM (pH 5.0). Change in absorbance of the reaction solution at 470 nm was determined after 1 min. One unit of pod activity was defined an absorbance change of 0.01 as U/min.

Thiobarbituric acid reactive substances (TBARS)

TBARS as an index of LPO was assessed according to the method used by (Iqbal *et al.*, 1996). The homogenized samples of both testis and sperm were separately mixed with 10 μL Tris–HCl buffer (150 mmol/L, pH, 7.1), 10 μL ferrous sulphate (1.0 mmol/L), 1 μL ascorbic acid (1.5 mmol/L) and 60 μL H_2O and incubated at 37 °C for 15 min. A volume of 1 mL of aqueous solution of trichloroacetic acid (10% w/v) was added to stop the reaction. An aliquot of 0.2 mL of thiobarbituric acid (0.37% w/v) was added and the sample was incubated at 100 °C for 15 min. Finally, the samples were centrifuged at 1000 ×g for 10 min. The amount of TBARS formed in each sample was estimated by measuring optical density at 532 nm. Results were expressed as nmol malonaldehyde/min/mg tissue and nmol malonaldehyde/min/10^8 spermatozoa at 37°C using a molar extinction coefficient of 156 mmol/L/cm.

Reactive oxygen species (ROS)

ROS in the homogenate was estimated according to the method described elsewhere (Hayashi *et al.*, 2007). Shortly, 5 μL homogenate or H_2O_2 (30 % w/w, Sigma Aldrich) standards prepared by serial dilutions of (0, 0.23, 0.46, 0.92, 1.87, 3.75 and 7.50 mg H_2O_2) were added to 140 μL of 0.1

mol/L sodium acetate buffer (pH, 4.8) in 96 well plate and incubated for 5 minutes at 37 °C. A volume of 100 µL of a mixed solution of N, N-diethyl-para-phenylenediamine (DEPPD) and ferrous sulfate (ratio 1:25) were added to each well and incubated for 1 min at 37 °C. Absorbance was taken at 505 nm using a microplate reader for 180 s with 15 s interval. Standard curve was plotted and concentrations of ROS in unit/g tissue and unit/108 sperm were reported. One unit of ROS was considered equivalent to levels of hydrogen peroxide in the sample (1 unit = 1.0 mg H_2O_2/L).

Assessment of DNA damage

Sperm DNA damage was assessed by using modified neutral comet assay according to the method used previously by (Boe-Hansen *et al.*, 2005). Sperm from cauda epididymis were collected in phosphate buffered saline (PBS) (pH 7.3) and were diluted to the concentration of 10^5 sperm/mL. Similarly, sperm from *in vitro* experiment was centrifuged at 1000 rpm for 10 min. The sperm pellet was diluted with phosphate buffered saline to a concentration of 10^5 sperm/mL. Shortly, a layer of regular melting point agarose was applied to the slides and cover slipped. Slides were placed at low temperature until the gel solidified. The coverslips were removed and a second layer of 85 µL low melting point agarose (65 µL of 1% low melting point agarose, 20 µL of sperm suspension) were spread on top of the first layer. Slides were coverslipped and allowed to solidify.

Lysis of cells was carried out by placing the slides in freshly prepared cold lysis buffer (pH 10.3, 2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris Base, 1% (w/v) Triton X - 100) for 24 hours. After three times washing with distilled water (20 min each), the slides were placed in an electrophoresis tray containing neutral electrophoresis buffer (54 g/L Tris base, 27.5 g/L boric acid, 0.5 mol/L EDTA, pH 7.4). Electrophoresis was performed for 20 min at 25V (0.71 V/ cm). The slides were air dried, wrapped in aluminum foil and kept at 5 °C overnight. The slides were rehydrated with distilled water, stained with acridine orange (300 - 400 µL of 20 mg/L of distilled water) and observed under an epifluorescent microscope (400 X, AFX - 1 Optiphot, Nikon, Tokyo, Japan). The numbers of comets/100 spermatozoa were counted and images were captured for scoring with comet assay score software (Tri Tek, V. 1.5). Number of comets, tail DNA (%) and tail moment (µm) were included in the results.

Hormonal Analysis

Determination of Testosterone

Testosterone concentrations were quantitatively determined in homogenate through Enzyme Linked Immuno Sorbant Assay (ELISA) kits (Amgenix, USA) following the guideline provided by the manufacturers.

Principle of the Test

The testosterone EIA is based on the principle of competitive binding between testosterone in the specimen and testosterone horseradish peroxidase (HRP) conjugate for a constant amount of rabbit anti-testosterone.

Procedure

To determine testosterone concentrations in the tissues, 10 µL of controls, standards and homogenate were added to the goat anti-rabbit IgG - antibody coated wells. A volume of 100 µL of testosterone-HRP conjugate reagent and 50 µL of rabbit anti-testosterone reagent were added to all the wells and incubated at 37 °C for 1.5 hour. The incubation is important for binding of testosterone in the sample and HRP-labelled testosterone with antibodies in the well. After incubation all the wells were rinsed 5 times with distilled water to remove unbound testosterone peroxidase conjugate. A volume of 100 µL of TMB reagent was added into each well, mixed for 10 seconds and incubated at room temperature for 20 minutes. After incubation 100 µL of stop solution was added to each well and mixed for 30 seconds. The absorbance was read at 450 nm using microplate reader. The results were calculated in ng/ml and were expressed in ng/g of tissue.in this assay 0.05 ng/mL was minimum detection limit for the kit and 6.4% was intra assay coefficients of variation.

Statistical analysis

Comparison of the values of control and treated groups was carried out by the one-way analysis of variance (ANOVA), followed by Dunnet's multiple comparison tests. A value of $P < 0.05$ was considered statistically significant. For all values, means ± standard errors of means (SEM) were calculated.

RESULTS

In vitro effects of BPA analogues BPB, BPF and BPS on testicular antioxidant enzymes, ROS and testosterone secretion in rat testis

In the testicular tissue antioxidant enzymes (CAT, POD, SOD), ROS and LPO were determined after incubations with different concentrations of BPA and its analogues BPB, BPF and BPS for two hours. No significant difference was observed in CAT, POD and SOD activity of any treated group as compared to control and presented in table 3.

ROS and LPO are considered oxidative stress markers were observed in *in vitro* treated groups of BPA and its analogues BPB, BPF and BPS and presented in table 3. LPO values have shown significant increase ($P < 0.05$) in BPS (100 ng/ml) treated group when compared to the control. However, other doses did not cause any significant increase in LPO values as compared to the control. ROS levels had shown significant decrease with increasing dose of BPA and its analogues BPB, BPF and BPS treated groups as compared to the control. Significant increase ($P < 0.05$ and $P < 0.01$) was observed in BPB and BPF (10 ng/ml) when compared to the control. ROS values were increased significantly ($P < 0.01$) in BPF (100 ng/ml) and BPS (100 ng/ml) treated groups. However, non-significant increase in ROS values was noticed as compared to the control group.

Decrease in testosterone concentration was observed after treatment of testis with BPA and its analogues BPB, BPF and BPS for two hours of incubation. All the doses of BPA and its analoguess caused non-significant decline in testosterone concentrations (Table 3).

Table 3. *In vitro* effects of bisphenol A and its analogues BPB, BPF and BPS (1, 10 and 100 ng/ml) on antioxidant enzymes and testosterone concentrations in rat testis

Treatments	Parameters					
	CAT (u/mg Protein)	POD (nmole)	SOD (u/mg protein)	LPO (min/mg Tissue)	Total ROS (U/g tissue)	Testosterone (ng/g tissue)
Control	8.12 ± 0.6	9.92 ± 2.7	10.51 ± 2.9	29.10 ± 0.2	25.8 ± 0.85	54.27 ± 0.4
BPA 1 ng/ml	7.18 ± 0.5	4.42 ± 0.6	7.18 ± 0.9	16.17 ± 1.5	33.6 ± 3.4	50.77 ± 4.7
BPA 10 ng/ml	3.94 ± 0.4	6.92 ± 1.1	13.27 ± 1.9	36.06 ± 2.8	27.6 ± 2.2	45.07 ± 2.0
BPA 100 ng/ml	6.54 ± 0.9	6.45 ± 1.3	13.40 ± 2.6	41.21 ± 4.8	34.6 ± 3.9	41.90 ± 0.2
BPB 1 ng/ml	2.79 ± 0.4	8.26 ± 2.5	11.75 ± 1.8	40.97 ± 5.3	33 .0± 3.3	42.59 ± 0.1
BPB 10 ng/ml	4.39 ± 0.7	9.86 ± 2.9	6.51 ± 1.2	40.91 ± 2.4	37.4 ± 2.5*	40.37 ± 3.1
BPB 100 ng/ml	6.90 ± 0.8	5.82 ± 0.8	14.25 ± 4.2	41.99 ± 4.5	36.8 ± 2.7	42.15 ± 3.7
BPF 1 ng/ml	4.64 ± 1.3	3.50 ± 0.6	13.96 ± 3.5	36.61 ± 7.0	34.8 ± 0.7	42.79 ± 0.4
BPF 10 ng/ml	6.63 ±1.0	5.41 ±1.3	11.00 ± 2.9	42.86 ± 5.3	43.8 ± 0.7***	42.37 ± 0.3
BPF 100 ng/ml	5.16 ±3.9	12.12 ± 4.2	10.49 ± 3.6	38.06 ± 6.1	41.2 ± 4.1**	41.46 ± 0.9
BPS 1 ng/ml	3.69 ±1.5	17.55±14.7	13.11 ± 1.7	37.05 ± 1.8	26.4 ± 2.9	53.15 ± 1.3
BPS 10 ng/ml	6.92 ± 3.9	11.73 ± 4.6	16.25 ± 0.8	42.17 ± 1.1	23.0 ± 0.7	51.65 ± 5.7
BPS 100 ng/ml	3.81 ± 0.8	7.24 ± 2.8	12.39 ± 0.8	52.49 ± 1.0*	39.8 ± 4.2**	52.00 ± 1.7

Values are expressed as mean ± SEM.

*, **, *** indicate significant difference at probability value $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control.

ANOVA followed by Dunnet's Comparison test.

In vitro sperm incubation with different concentrations of BPA and its analogues BPB, BPF and BPS

Superoxide dismutase (SOD) and Lipid peroxidation and (LPO)

Antioxidant activities of SOD and LPO were determined after incubation of sperms with different concentrations of BPA and its analogues BPB, BPF and BPS. SOD levels showed significant increase ($P < 0.05$) in BPA 100 µg/L (5.65 ± 0.29 mU/10^8 Spermatozoa) as compared to the control (3.71 ± 0.10 mU/10^8 Spermatozoa). Significant increase was observed in BPB 100 µg/L ($P < 0.01$) when compared to the control group. Similarly, BPF and BPS (100 µg/L) also caused significant increase ($P < 0.01$ and $P < 0.05$) when compared to control group. On the other hand, non-significant increase in the total SOD levels was noticed as shown in fig 5.

TBARS levels in different treatment groups and control is presented in fig 6. Significant increase was observed in BPA 100 µg/L ($P < 0.01$) when compared to control. The measured TBARS concentrations in 100 µg/L BPA treated group was 1.13 ± 0.04 nmol malonaldehyde/ 10^8 spermatozoa, while in control group it was as 0.63 ± 0.03 nmol malonaldehyde/10^8 spermatozoa. Similarly, in both BPB and BPF 100 µg/L groups significant increase ($P < 0.05$) was observed in treatment groups as compared to control. TBARS levels increased significantly ($P < 0.05$) in BPS 100 ug/L treated group as compared to control. The values of other treatment groups of BPA and its analogues BPB, BPF and BPS had increased but that difference was not significant as compared to control group.

Reactive oxygen species (ROS)

Oxidative stress was checked in the samples by measuring ROS in the treated groups with BPA and its analogues BPB, BPF and BPS and presented in fig 7. In control group, values of ROS were 0.02 ± 0.005 unit/10^8 spermatozoa which were lower to the spermatozoas exposed 100 µg/L (0.044 ± 0.005) of BPA. While the values of BPB and BPF also increased significantly ($P < 0.05$) compared to control and the values in these two groups were 0.038 ± 0.003 and 0.007 ± 0.003. Similarly, ROS Levels also increased ($P < 0.05$) in treated group with BPS 100 µg/L as compared to control. There was no significant change observed in ROS levels of BPA and its analogues BPB, BPF and BPS treated groups 1-10 µg/L in comparison to the control group.

DNA damage in the rat spermatozoa

DNA damage in the spermatozoa was measured by comet assay and is presented in table 4. The underlying principle of comet assay is the ability of denatured DNA fragments to migrate during electrophoresis. Electrophoresis can be carried out under highly alkaline conditions (pH > 12.6) in order to detect single-strand and double-strand breaks and alkali-labile lesions. The results show non-significant difference in DNA fragmentation in spermatozoa nuclei of BPA and its analogues BPB, BPF and BPS (1-10 µg/L) treated groups as compared to the control after 2 hours of incubation but there was significant ($P < 0.05$) increase observed in the DNA fragmentation in spermatozoa of BPA and its analogues BPB, BPF and BPS groups of 100 µg/L as compared to control.

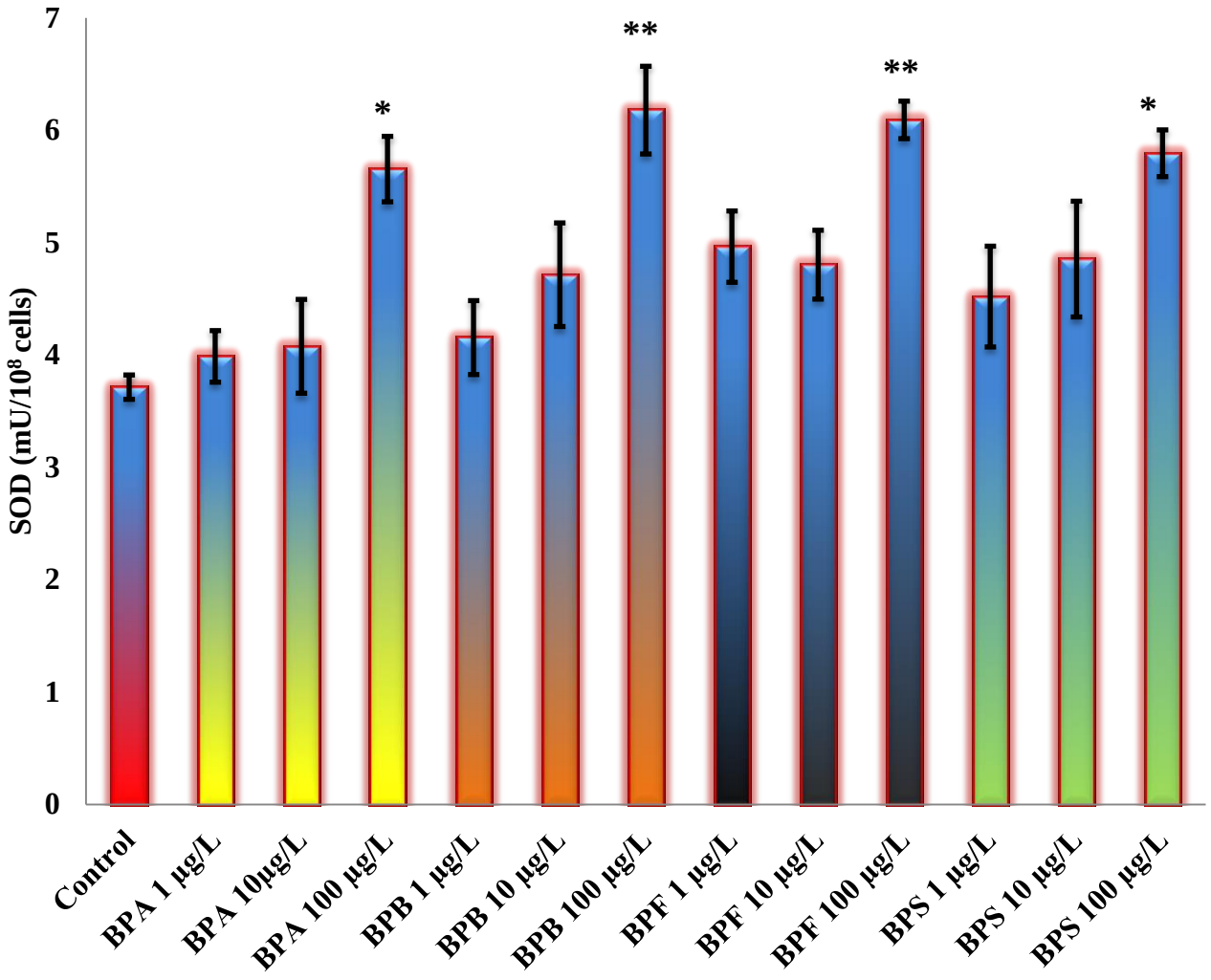

Fig 5 *In vitro* effect of BPA, BPB, BPF and BPS on SOD activity in rat sperms after 2 hours of incubation. SOD activity measured in control and BPA, BPB, BPF and BPS (1, 10, and 100 µg/L) treated groups. Results are expressed as mean ± SEM (n = 7 for each condition) and presented as SOD (mU/10^8 cells). Significant results (P < 0.05 and P < 0.01) are indicated: *, ** versus control.

P < 0.05 *

P < 0.01 **

P < 0.001 ***

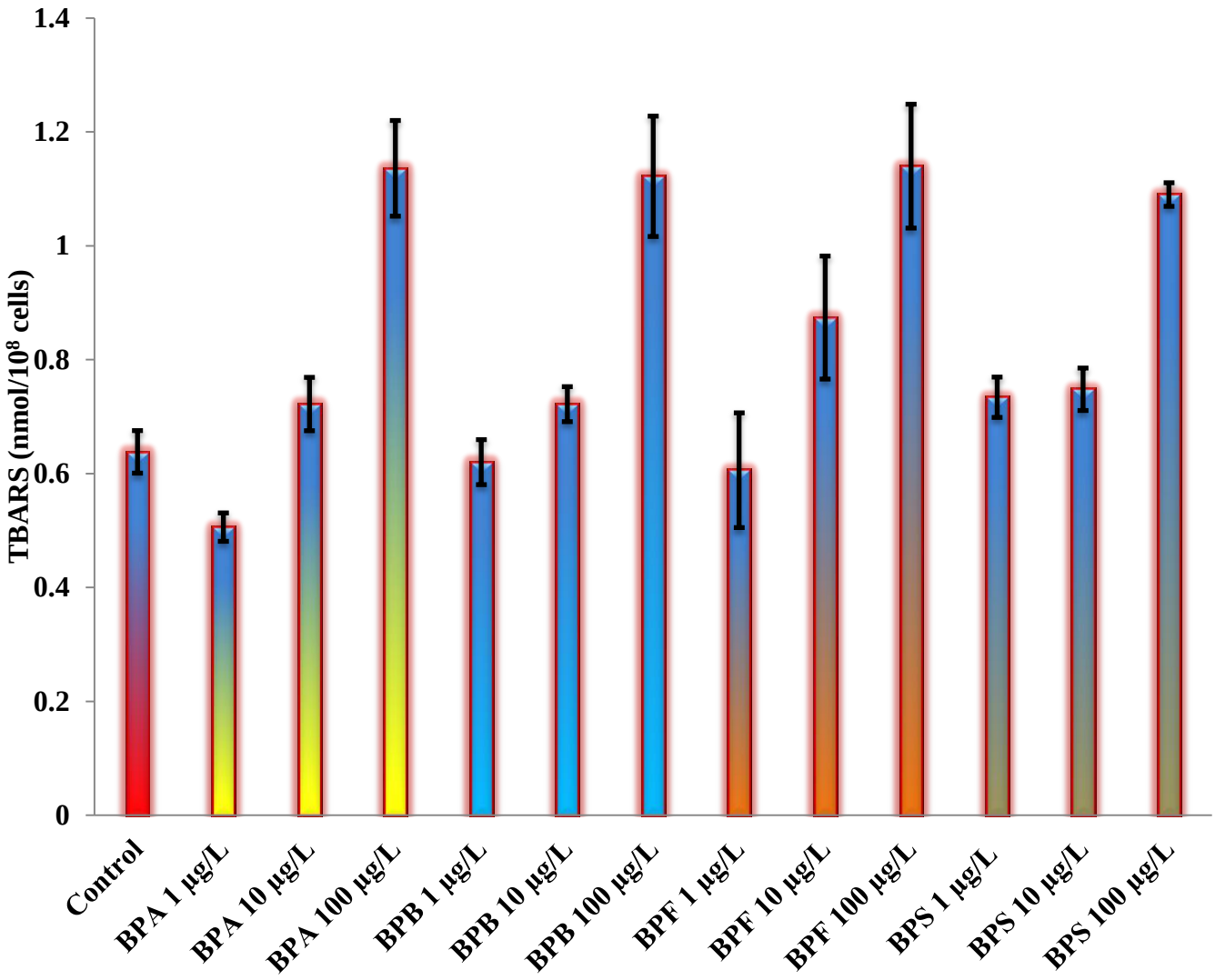

Fig 6. Effects of BPA, BPB, BPF and BPS on TBARS in rat sperm after 2 hours of incubation *in vitro*. TBARS measured in control and BPA, BPB, BPF and BPS (1, 10, and 100 µg/L) treated rat sperm groups are expressed as mean ± SEM (n = 7 for each condition) and presented as TBARS (nmol malonaldehyde/10^8 cells). Significant results (P < 0.01 and P < 0.01) are indicated: *, **versus control.

P < 0.05 *

P < 0.01 **

P < 0.001 ***

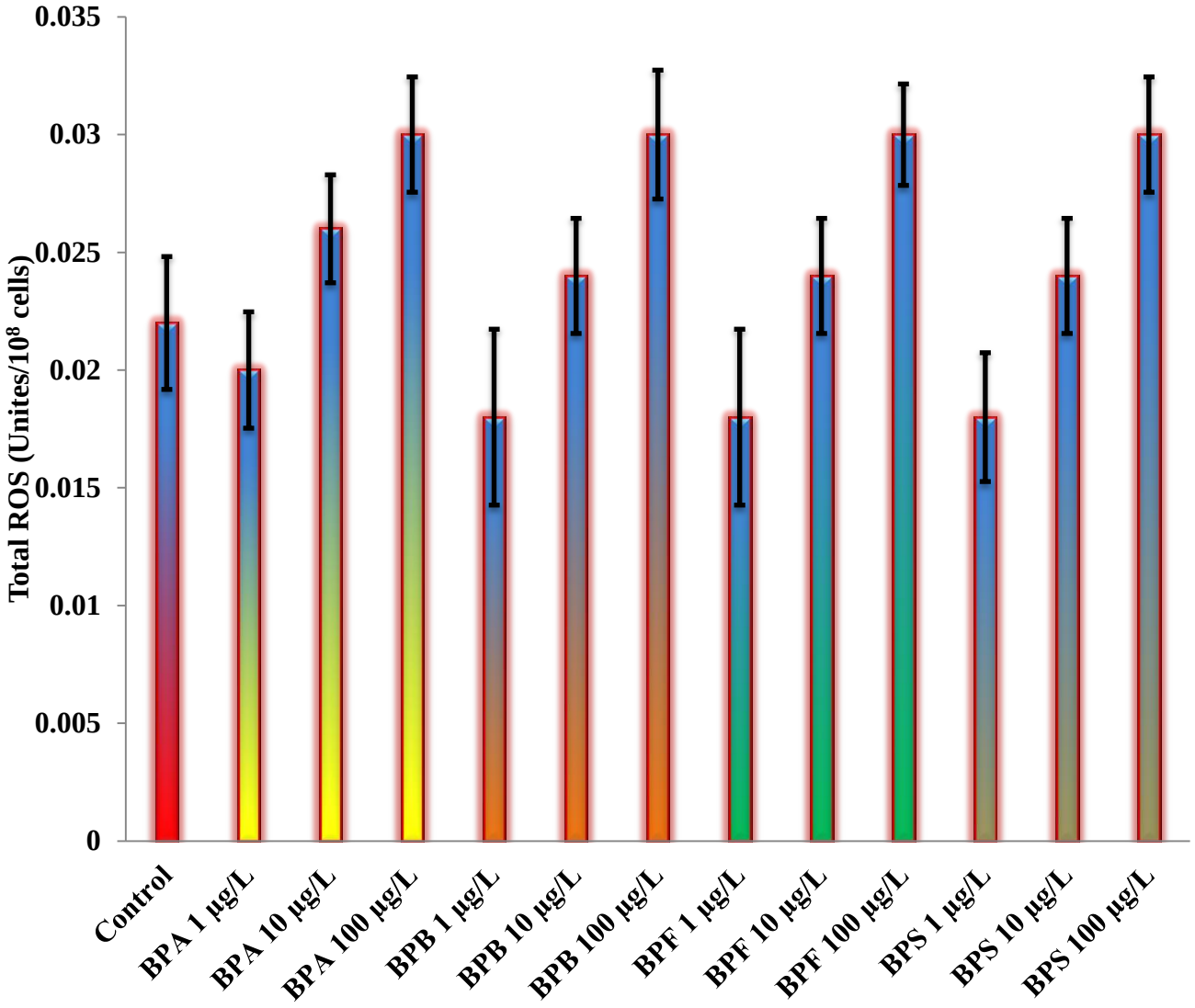

Fig 7. Effects of BPA and its analogues BPB, BPF and BPS on ROS in rat sperm after 2 hours of incubation *in vitro*. ROS measured in control and BPA, BPB, BPF and BPS (1, 10, and 100 µg/L) treated rat sperm groups are expressed as mean ± SEM (n = 7 for each condition) and presented as ROS (units/10^8 cells). Significant results ($p < 0.05$) are indicated: *versus control

P < 0.05 *

P < 0.01 **

P < 0.001 ***

Table 4. Mean ± SEM of rat sperm DNA damage in control and sperms incubated with different concentrations of BPA, BPB, BPF and BPS (1, 10, and 100 ug/L) for 2 hours

	Parameters		
Groups	**Number of comets/100 cells**	**Tail moment (µm)**	**Tail DNA (%)**
Control	14.62 ± 0.16	6.33 ± 0.15	14.54 ± 0.25
BPA 1 ug/L	15.08 ± 0.31	6.97 ± 0.30	14.02 ± 0.28
BPA 10 ug/L	13.97 ± 0.16	7.78 ± 0.18	15.96 ± 0.34
BPA 100 ug/L	16.41 ± 0.30*	8.44 ± 0.70*	17.34 ± 0.21*
BPB 1 ug/L	13.57 ± 0.60	6.92 ± 0.16	15.12 ± 0.28
BPB 10 ug/L	14.79 ± 0.20	7.52 ± 0.22	15.82 ± 0.18
BPB 100 ug/L	16.06 ± 0.72*	8.16 ± 0.36*	17.12 ± 0.16*
BPF 1 ug/L	13.81 ± 0.12	6.16 ± 0.16	13.98 ± 0.22
BPF 10 ug/L	14.35 ± 0.14	8.24 ± 0.13	14.92 ± 0.22
BPF 100 ug/L	16.58 ± 0.82*	8.06 ± 0.24*	17.51 ± 0.18*
BPS 1 ug/L	13.48 ± 0.16	6.41 ± 0.17	13.82 ± 0.30
BPS 10 ug/L	14.83 ± 0.26	7.06 ± 0.55	14.62 ± 0.34
BPS 100 ug/L	16.65 ± 0.60*	8.48 ± 0.22*	16.98 ± 0.06*

Values are expressed as mean ± SEM.

* indicates significance at $p < 0.05$ vs. control.

DISCUSSION

BPA analogues BPB, BPF and BPS have been used in the modern world as alternative to BPA in BPA free daily use items (Rosenmai *et al.*, 2014). Data from many agencies of environment monitoring has shown that these chemicals are going to become a serious threat to both human and animal life. Many environment protection experts say that BPA and its analogues BPB, BPF and BPS and are going to become the most concern environmental pollution and food contaminant in future (Liao and Kannan, 2013, Qiu *et al.*, 2018b, Mu *et al.*, 2018b). Present investigation revealed significant increase in the production of ROS and LPO concentrations which are an indication of oxidative stress in the *in vitro* study.

BPA and other phenolic compounds have been proven to be generating ROS in many studies (Kourouma *et al.*, 2015, Lee *et al.*, 2013, Huc *et al.*, 2012, Hulak *et al.*, 2013). Oxidative stress also affects the function of sperm by damaging lipids which is in the form of poly unsaturated fatty acids concentration in the sperm plasma membrane (Ullah *et al.*, 2017, Zalata *et al.*, 2004, Ullah *et al.*, 2018). Our results showed that SOD levels were high in sperm samples incubated with 100 µg/L BPA and its analogues BPB, BPF and BPS as compared to the control group, which seem to have been induced due to activation of body defense mechanism of antioxidant enzymes to reutilize free radicals generated by ROS. In the previous studies, it was also reported that BPS incubation for 2 hour increase the levels of SOD in testicular tissues (Ullah *et al.*, 2016, Ullah *et al.*, 2017, Ullah *et al.*, 2018). Some other studies on BPA and BPS exposure also increased the levels of SOD by inducing oxidative stress in the sperm and reproductive tissues (Hulak *et al.*, 2013, Potts *et al.*, 2000, Ullah *et al.*, 2017). This led to the formation of ROS and high levels of lipid peroxidation in the testicular tissues. In the present study the levels of ROS and T-BARS were also observed high in the groups treated with BPA and its analogues BPB, BPF and BPS 100 ug/L as compared to the control. Damage to the sperm in groups treated with 100 ug/L of BPA, BPB, BPF and BPS resulted in an increased oxidative stress and high levels of ROS. Previous *in vitro* studies can support our results by (Ullah *et al.*, 2017, Liang *et al.*, 2016, Lee *et al.*, 2013, Ullah *et al.*, 2018) in mice, chicken and rats. In the previous studies, BPS and BPA exposure also increased the levels of SOD by induceing oxidative stress in the sperm and reproductive tissues (Manfo *et al.*, 2014, Maćczak *et al.*, 2017, Dong *et al.*, 2018, Rhee and Rhee, 2016).

In vitro study conforms sperm DNA damage in all the treated groups of BPA and some its analogues as shown in the previous studies where BPA and some of its analogues exposure caused reduction in the testosterone concentrations, increased levels of estrogen, reduce number of eggs and pups and modified transcripts of GnRH (Ji *et al.*, 2013, Ullah *et al.*, 2017, Feng *et al.*, 2012, Roelofs *et al.*, 2015, Ullah *et al.*, 2018). BPA has also been observed to mimic estrogen receptor alongside with anti-androgenic effects resulting in the suppressed plasma and intra-testicular testosterone concentrations (Sakaue *et al.*, 2001, Grignard *et al.*, 2012, Ullah *et al.*, 2017, Ullah *et al.*, 2016, Ahsan *et al.*, 2018b, Ullah *et al.*, 2018). On the basis of the above previous studies we can say that the anti-androgenic effects of BPA and its analogues BPB, BPF and BPS led to DNA damage in sperm, high levels of ROS and LPO, which resulted in the altered hormonal concentrations in the present study.

CONCLUSIONS

In the present study, antioxidant enzymes status of the testicular tissues was depleted and oxidative stress was induced in the reproductive tissues after exposure to BPA and its analogues BPB, BPF and BPS. Some of the BPA analogues have also been reported to have genotoxic effects and in the *in vitro* studies they have also induced apoptosis (Mokra *et al.*, 2015, Rahman *et al.*, 2015, Barbonetti *et al.*, 2016, Yin *et al.*, 2016). The present *in vitro* study results suggest that BPA and its analogues BPB, BPF and BPS exposure altered the process of spermatogenesis, caused changes in the antioxidant enzymes activity and sperm DNA damage which suggest the relation of sperm motility and DNA damage.

ABSTRACT

Background: Bisphenol A (BPA) is used for the production of plastic products and epoxy resins. World-wide exposure of BPA led regulations in its production and was banned by many countries in 2010 in many of daily use items mainly as baby feeding bottles. This ban led to the production of many analogues of BPA known as BPA alternatives. BPA analogues as bisphenol B (BPB), bisphenol F (BPF) and bisphenol S (BPS) have similar chemical structure and binding ability for estrogen receptor (ER) as BPA.

Materials and methods: Based on *in vitro* results from our previous study, an *in vivo* study was done to determine comparative toxicity of BPA, BPB, BPF and BPS in male rats. Prior to the sub-chronic study an acute toxicity testing was performed using female rats according to the guidelines of organization for economic cooperation and development (OECD) protocol 407. Healthy female adult rats (n=5/group) were orally administered with different concentrations of BPA and its analogues BPB, BPF and BPS (5, 50, 300 and 2000 mg/kg). All the exposed animals were checked for mortality and signs of toxicity for the next 14 days. In the present study BPA and its analogues BPB, BPF and BPS comparative toxicity exposure was evaluated on testosterone concentrations, oxidative stress and antioxidant enzymes activity in reproductive tissues. In the *in vivo* study, adult male rats were exposed to different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) for 28 days.

Results: In the acute toxicity study, no mortality or signs of toxicity were observed during observation period of 14 days. In the *in vivo* study, we observed that antioxidants enzymes and protein content was reduced while reactive oxygen species (ROS) and lipid profile was elevated in treated groups as compared to the control. Plasma testosterone concentrations and intra-testicular testosterone in testis was reduced in the treated groups as compared to control. On the other hand, histopathology study also revealed degenerative changes in the morphology of testis treated with different concentrations of BPA and its analogues as compared to control. The present comparative study on BPA and its analogues BPB, BPF and BPS suggest the toxic effect of these chemicals on testis and spermatogenesis and we also observed that these chemicals induce oxidative stress in the reproductive tissues of male rats.

Conclusion: The present comparative study on BPA and its analogues BPB, BPF and BPS suggest the toxic effects of BPA and its analogues BPB, BPF and BPS on testis and spermatogenesis and we also observed that BPA and its analogues also induce oxidative stress in the reproductive tissues of male rats.

INTRODUCTION

BPA has a very long story in the history of sciences. In 1936, for the first time its estrogenic properties were reported in the reproductive system of female rats (Dodds and Lawson, 1936). BPA was introduced into industry for the manufacture of polymers synthesis (Epoxy resins, polycarbonate and certain plastics) (Scippo, 2011). BPA has been used as the main component of many consumer products like infants feeding bottles, coatings of beverages, food cans, medical devices and dental sealants (Scippo, 2011). Depending upon exposure to temperature and pH, BPA can migrate into environment and has been detected in saliva, blood and food (Scippo, 2011, Calafat *et al.*, 2009, Calafat *et al.*, 2008, Braun *et al.*, 2011a, Van Landuyt *et al.*, 2011, Ahn *et al.*, 2008). As a weak estrogen BPA has two OH and benzene rings which fit into the binding pocket of estrogen receptor (ER) (Kuiper *et al.*, 1998). BPA binding affinity makes it a classical ligand for both ER α and ER β receptor which also increase the estrogen receptor potency (Wetherill *et al.*, 2007).

In response to American chemistry council (ACC) in 2012, food development authority (FDA) banned the use of BPA in sippy cups and infants feeding bottles. This ban resulted in introduction to BPA alternatives such as BPB, BPF and BPS (Liao and Kannan, 2014b). Since BPA analogues have similar chemical structure and binding ability for estrogen receptor (ER) they have also shown toxicological effects (Chen *et al.*, 2002a, Nunez *et al.*, 2001). BPB is an analogue of BPA used for the manufacturing of phenolic resins (Cunha and Fernandes, 2010) and is found in 21.4% samples of food from Italian supermarkets (Cunha and Fernandes, 2010) and 0.88 to 11.94% in endometriosis of women and 27.6% in the sera (Liao *et al.*, 2012b, Cobellis *et al.*, 2009). BPA is also found in indoor dust (Liao *et al.*, 2012d, Liao *et al.*, 2012b) however, there is very limited data on human exposure of BPB. Previously in a study of 20 tested human urine samples around two were shown positive with BPB (Cunha and Fernandes, 2010). BPB has estrogenic effects and is more resistant to biodegradation (Li *et al.*, 2014a, Ike *et al.*, 2006) causing decrease in cortisol and corticosterone levels which also lead to DNA damage (Rosenmai *et al.*, 2014, Chen *et al.*, 2002a). Another member of BPA family BPF has a lot of implications and is used in the manufacturing of polycarbonates and epoxy resins (Liao and Kannan, 2014b, Molina-Molina *et al.*, 2013). Several studies have shown BPF presence in the stuffed food and in drinking water pumped through BPF used pipes (Cabado *et al.*, 2008, Zou *et al.*, 2012). BPF was also found in meat products, beverages and vegetables available in the

supermarkets (Gallart-Ayala *et al.*, 2011b, Liao and Kannan, 2013). BPF has been found in different organs of the body and can cross placental barrier to reach the fetus (Cabaton *et al.*, 2006).

BPA another replacement BPS was firstly synthesized as a dye in 1869 which came into use after the ban on BPA in epoxy resins, infant feeding bottles and thermal papers in 2006 (Glausiusz, 2014, Liao *et al.*, 2012c). There have been several studies where BPS has been detected in 81% human matrices and in 3% of breast milk (Bergmann *et al.*, 2015, Chen *et al.*, 2002a, Fic *et al.*, 2013b, Rochester and Bolden, 2015). The analogues of BPA such as BPB, BPF and BPS have genotoxic effects and also induce oxidative stress in different tissues. The endocrine disruptive nature of these analogues proposes that they are more harmful than BPA and are not safe alternatives for BPA (Feng *et al.*, 2016). BPA analogues have toxic effects including cytotoxicity, reproductive toxicity, neurotoxicity and endocrine disruption reported by several studies. A study on BPA analogues showed that BPS and BPF have similar potency for androgenic, antiandrogeic, estrogenic and antiestrogenic receptor as compared to BPA (Liao *et al.*, 2012d). In the present study comparative effects of BPA and its analogues BPB, BPF and BPS were determined in reproductive system of male rats in a sub chronic toxicity study.

MATERIALS AND METHODS

Experimental animals

Sprague Dawley adult male rats (age 80 to 90 days) were obtained from the Primate/Rodent Facility of Quaid-i-Azam University Islamabad, Pakistan. Animals were housed in steel cages and each cage contained a maximum of 5 animals. Prior to the start of the experiment standard laboratory conditions were maintained. Room temperature was maintained at 22-25 ℃ and light/dark cycle was maintained. Throughout the experimental period animals were fed with laboratory feed and tap water was available freely for the animals. Protocols of handling of the animals were approved by Ethical Committee of Department of Animal Sciences, Quaid-i-Azam University Islamabad, Pakistan.

Experimental design

Different experiments were designed to investigate the comparative effects of BPA and its analogues BPS, BPB and BPF exposure on the male reproductive system. Our first *in vitro* experiment in which the direct effect of BPA and analogues BPB, BPF and BPS on the concentration of testosterone and levels of antioxidant enzymes in testis an acute oral toxicity study was performed before the sub chronic exposure study. In the second study different concentrations of BPA analogues BPB, BPF and BPS on reproductive system of male rats were determined through twenty eight days sub chronic exposure study.

Acute oral toxicity study of BPA and its analogues BPB, BPF and BPS in rats

Adult female Sprague Dawley rats (80 to 90 days Old, n=85) were used in the acute oral toxicity study. Animals were divided into seventeen groups (n=5/group). All the female rats were dosed according to the OECD protocol 407 for the acute toxicity test of the chemicals. Acute toxicity study was performed to evaluate the toxic effects of BPA and its analogues BPB, BPF and BPS on female rats because female animals are more sensitive to the toxicity compared to male animals. BPA and its analogues BPB, BPF and BPS all groups were treated with four different concentrations of (5, 50, 300 and 2000 mg/kg) for fourteen consecutive days while control group received saline with 1% ethanol. Animals were checked for appearance of any sign of toxicity and mortality for the first 24 hours with special attention during the first four hours. Animals were further observed for the next 72 hours with special attention and daily till the completion of the fourteen days experiment. On day fourteen, all the animals were euthanized and the mortality data was reported.

***In vivo* sub chronic toxicity study on BPA and its analogues BPB, BPF and BPS**

Adult male Sprague Dawley rats (70 to 80 days old; n = 91) were divided into thirteen groups (n=7/group). Animals were exposed for twenty-eight consecutive days orally to different concentrations (5, 50 and 500 mg/kg body weight/ day) of BPA and its analogues BPB, BPF and BPS. Ethanol was used for the preparation stock solutions of BPA and its analogues later the stock solutions were diluted in saline where final concentration of ethanol was 0.1 to 0.5%. Animals were euthanized on twenty-ninth day of the sub chronic study and blood and reproductive organs were collected for the different histological and biochemical tests. Testicular tissues (left testis and left epididymis) were weighed and processed for biochemical analysis and (right testis and right epididymis) were placed in 10 % buffer formalin for the histopathology. Blood collected from the animals was centrifuged at 3000 rpm for 10 min and plasma was separated and stored at -20 ℃ until biochemical and hormonal analysis.

Biochemical and histopathological analysis

As the animals were euthanized and different reproductive tissues were removed and preserved for different biochemical and histological analysis.

Antioxidant enzymes

Different antioxidant enzymes assays and oxidative stress markers activities were carried out as explained in detail in chapter 1.

Total protein content

AMEDA Laboratory diagnostic kits from GmbH Krenngasse, Graz/Austria were used for the determination of total protein in tissues. The results of protein were measured by plotting absorbance of the standard against samples.

Sperm motility

Immediately after dissection, cauda epididymis was cut slightly with a scissor in 0.5 mL pre-warmed (at 37 ºC) phosphate buffer saline (PBS) (pH 7.3) containing a drop of nigrosin stain. An aliquot of 50 µL was taken and placed on pre-cleaned and warmed (at 37 ºC) glass slide and was observed under a light microscope at 40X. A total of 100 sperm/sample were analyzed for motility by a technician blinded to the treatment groups. Each sample was analyzed three times and the average value was used as the total sperm motility.

Sperm count and daily sperm production (DSP)

DSP was done in the testicular tissues with the help of rotostaor homogenizer (IKA-Werke, Staufen, Germany) the thawed samples were homogenized in 5 ml of solution which contained 0.5% NaCl and 5% triton X-100. The homogenized sample was diluted and transferred to neubar chamber and 19th stage spermatids were counted under microscope at 40X. Sperm count was done in the testicular tissues as the obtained values by the sperm count in the testes were divided by 6.3 (number of days the spermatids remain in seminiferous epithelium).

Daily sperm production (DSP) = Y / 6.3

Hormonal Analysis

Plasma and intra-testicular testosterone concentrations were determined by the method mentioned in chapter 1.

Tissue Histology

Testicular and epididymis histology was carried out in order to determine the BPA and its analogues BPB, BPF and BPS Toxicity. After removal of testicular and epididymal tissues the following steps were performed.

Fixation

Testis and epididymis were fixed in 10% formaldehyde for 48 hours.

Dehydration

Following fixation, dehydration was carried out at room temperature in the following ascending grades of alcohol

70% Ethanol …….. 2 hr

80% Ethanol …….. 2 hr

90% Ethanol …….. 2 hr

100% Ethanol …… 3 changes (2 hrs each)

After dehydration fixed tissues were transferred to xylene (2 changes, 2 hrs each) to become clear and transparent at room temperature.

Embedding

Tissue were transferred to paraplast for embedding according to the following

Benzol + Paraplast (1:1) …… 2 hrs (at 60 ^{0}C)

Paraplast 1 ……. 4 hrs (at 62 ^{0}C)

Paraplast 2 ……..... 4 hrs (at 62 ^{0}C)

Paraplast 3 …….. 4 hrs (at 62 ^{0}C)

After the above process, tissues were transferred in a boat containing melted wax. Wax was allowed to solidify after removing bubbles from it. With the help of knife or scalpel paraffin wax blocks were trimmed and mounted on wooden blocks for section cutting.

Tissue sectioning

Sections 7 µm thickness was cut out of the tissue using microtome (Thermo, Shandon finesse 325, UK). The ribbons with tissues were stretched and fixed to previously clean albumenized glass slides on Fischer slide warmer (USA) at 60 ^{0}C. These glass slides were placed in incubator (45 ^{0}C) overnight for completion of stretching and removal of bubbles any left.

Staining

For staining following steps were followed

Hydration of sections

The slides were deparafinized in xylene (two changes, 10 min each) and the sections were rehydrated in the descending grades of ethanol.

100% ethanol ---------- 2 changes (3 min each)

90% ethanol ---------- 2-5 min

70% ethanol ---------- 2-5 min

50% ethanol ---------- 2-5 min

30% ethanol ---------- 2-5 min

Water ---------- 2-5 min

Hematoxylin ---------- 5-8 min

Wash in tap water ---------- 5-10 minutes until the tissue turned blue.

95 % acidified alcohol ------ 2-3 min

Bluing solution (water + few drops of NH4OH) -------- 1 min

Washing with tape water ----- 2 min

70% ethanol ---------- 2 min

Eosin ---------- 2 min washing in tape water ------ 1 min

90% ethanol ---------- 2 min

100% Alcohol ---------- 2-5 min

Xylene ---------- 2 changes (5 min each)

Two to three drops of canada bolsom was put on the slides and were cover slipped.

Microscopy

Prepared slides were observed under Leica Microscope (New York, USA) equipped with digital camera (Canon, Japan). Images were taken at 20X and 40X and morphometry was done using software Image J.

Area of the seminiferous tubules, epididymis tubules and interstitial space was determined by planimetry, using Image J software. Area in μm2 was calculated according to (Ullah *et al.*, 2016). Shortly 25 pictures at 20X per animal of known area were selected and the area of seminiferous tubules, epididymis tubules and interstitial space was determined by free selection tool of the software. The area % age was calculated by the formula %As=As*100/T

Where As is area covered by seminiferous tubules and T is total area of the field.

Percentage of the mean area was analyzed for comparison between treated groups and control and was reported. Percentage of the mean area was analyzed for comparison between treated groups and control and was reported. Number of different cell types was counted from fifty seminiferous tubules per animal at 100X, and mean number of spermatogonia, spermatocytes and spermatids per seminiferous tubule were reported.

Statistical analysis

The Dunnett's multiple comparison tests, which followed analysis of variance (ANOVA), were used for the comparison of different groups with the control using GraphPad Prism software. Values were expressed as mean ± SEM and were considered significant at $P < 0.05$.

RESULTS

BPA and its analogues BPB, BPF and BPS acute toxicity in rats

BPA and its analogues BPB, BPF and BPS acute toxicity in rats after single oral dose (5, 50, 500 and 2000 mg/kg body weight) is presented in table 5. After duration of 14 days there was no sign of toxicity or mortality observed in the animals treated with different concentrations of BPA and its analogues BPB, BPF and BPS. There was only increase observed in the breathing of some animals after treatment with high doses of BPA and its analogues BPB, BPF and BPS.

Effects of BPA and its analogues BPB, BPF and BPS on body weight gain and testicular weight after sub-chronic administration

Body weight gain after 28 days of exposure, showed no significant change in all the treated groups as compared to the control. Similarly, in the left and right testis of all the treated groups no significant change was observed when compared to the control (Table 6).

Biochemical parameters of rat testis after sub chronic treatment with BPA and its analogues BPB, BPF and BPS

Antioxidants enzymes, SOD and POD in the testicular tissues after 28 days of sub chronic exposure are presented in table 7. There was no significant change observed in the SOD activity when treated groups were compared to the control. However, in the POD activity significant reduction was observed in BPA 50 mg/kg ($P < 0.001$) when compared to the control. POD activity was reduced significantly ($P < 0.01$, $P < 0.01$ and $P < 0.05$) in BPB 5, 25 and 50 mg/kg treated groups. Similarly, BPF treatment caused significant reduction ($P < 0.01$, $P < 0.05$ and $P < 0.01$) at dose level of 5, 25 and 50 mg/kg. On the other hand, PBS 5 mg/kg significantly ($P < 0.05$) reduced POD in the testicular tissues; however, the other doses of BPS did not reduce POD level as compared to the control.

Activity of CAT in the testicular tissues after 28 days of exposure showed significant reduction in BPA 5 and 25 mg/kg ($P < 0.05$ and $P < 0.01$) as compared to the control. BPB 25 mg/kg caused significant reduction ($P < 0.05$) in CAT activity of testicular tissues when compared to control. Similarly, BPF 25 and 50 mg/kg reduced ($P < 0.01$) CAT values as compared to control. On the other hand, BPS 5 and 50 mg/kg significantly reduced ($P < 0.01$ and $P < 0.05$) CAT

activity in testicular tissues. However, no significant difference was observed in other treated groups as compared to the control group.

LPO, a well-known oxidative stress marker was determined in the reproductive tissues of male rats and is presented in table 7. Significant increase in the LPO (T-BARS) content was observed in BPA 50 mg/kg ($P < 0.05$) when compared to control. LPO content reduced significantly ($P < 0.01$) in BPB 50 mg/kg treated group as compared to the control group. Similarly, BPF treatment caused significant reduction ($P < 0.001$) at dose level of 50 mg/kg, however, BPF 5 and 25 mg/kg did not affect POD activity. BPS 50 mg/kg significantly reduced ($P < 0.01$) LPO activity in the testicular tissues. However, the other doses of BPS did not show significant effect as compared to control.

Total ROS in the different treatment groups and control is presented in table 7. Significant increase was observed in BPA 50 mg/kg ($P < 0.001$) group when compared to control. Total ROS was also increased significantly ($P < 0.001$) in BPB 50 mg/kg treated group when compared to control. Similarly, BPF and BPS treatments caused significant increase ($P < 0.01$, $P < 0.001$ respectively) at dose level of 50 mg/kg as compared to the control group. However, total ROS was not altered by BPS 5 and 25 mg/kg in comparison to the control group.

Total protein in the testis after 28 days of exposure showed significant reduction in BPA 5 mg/kg ($P < 0.05$), BPA 25 mg/kg ($P < 0.01$) and BPA 50 mg/kg ($P < 0.05$) as compared to the control. Protein concentration was reduced significantly ($P < 0.05$) in BPB 5 and 50 mg/kg treated groups as compared to the control. On the other hand, BPF 5 and 25 mg/kg treatment groups showed significant reduction ($P < 0.05$, $P < 0.001$) in protein levels as compared to control. Similarly, BPS 5, 25 and 50 mg/kg reduced total protein as compared to control presented in table 7.

Table 5. Acute toxicity of different concentrations of bisphenol A and its analogues BPB, BPF and BPS (0, 5, 50, 300 and 2000 mg/kg) after single oral dose in female rats

Test sequence	Dose (mg/kg)	No of animals	After (24 h)	After (72 h)	After (14 days)
Control	0	5	Survival	Survival	Survival
BPA 1	5	5	Survival	Survival	Survival
BPA 2	50	5	Survival	Survival	Survival
BPA 3	300	5	Survival	Survival	Survival
BPA 4	2000	5	Survival	Survival	Survival
BPB 1	5	5	Survival	Survival	Survival
BPB 2	50	5	Survival	Survival	Survival
BPB 3	300	5	Survival	Survival	Survival
BPB 4	2000	5	Survival	Survival	Survival
BPF 1	5	5	Survival	Survival	Survival
BPF 2	50	5	Survival	Survival	Survival
BPF 3	300	5	Survival	Survival	Survival
BPF 4	2000	5	Survival	Survival	Survival
BPS 1	5	5	Survival	Survival	Survival
BPS 2	50	5	Survival	Survival	Survival
BPS 3	300	5	Survival	Survival	Survival
BPS 4	2000	5	Survival	Survival	Survival

Table 6. Effects of sub-chronic exposure of different concentrations of Bisphenol A and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) on the different parameters of rat testis

Treatments	Parameters				
	Body weight gain (g)	**Right Testis weight (g)**	**Left testis weight (g)**	**SOD (u/mg protein)**	**POD (nmole)**
Control	**33 ± 4.11**	**1.06 ± 0.05**	**1.13 ± 0.02**	**48.54 ± 1.51**	**15.15 ± 0.20**
BPA 5 mg/kg	**25 ± 3.81**	**1.16 ± 0.73**	**1.16 ± 0.08**	**26.29 ± 5.71**	**14.77 ± 0.68**
BPA 25 mg/kg	**22 ± 3.21**	**1.02 ± 0.07**	**1.02 ± 0.04**	**20.38 ± 4.38**	**13.30 ± 0.96**
BPA 50 mg/kg	**22 ± 4.10**	**1.11 ± 0.08**	**1.04 ± 0.05**	**38.26 ± 8.20**	**11.95 ± 0.22*** **
BPB 5 mg/kg	**29 ± 3.22**	**1.21 ± 0.05**	**1.16 ± 0.05**	**25.96 ± 11.42**	**12.55 ± 0.49** **
BPB 25 mg/kg	**23 ± 3.81**	**1.12 ± 0.05**	**1.12 ± 0.06**	**32.54 ± 3.38**	**13.11 ± 0.59***
BPB 50 mg/kg	**26 ± 4.01**	**1.12 ± 0.06**	**1.16 ± 0.05**	**29.81 ± 8.12**	**12.81 ± 0.28***
BPF 5 mg/kg	**27 ± 2.71**	**1.01 ± 0.06**	**1.04 ± 0.07**	**21.32 ± 3.87**	**12.55 ± 0.43** **
BPF 25 mg/kg	**23 ± 2.23**	**1.12 ± 0.04**	**1.14 ± 0.09**	**33.34 ± 7.42**	**12.85 ± 0.09***
BPF 50 mg/kg	**27 ± 3.23**	**0.97 ± 0.11**	**1.26 ± 0.05**	**36.02 ± 10.65**	**12.61 ± 0.39** **
BPS 5 mg	**25 ± 4.10**	**1.18 ±0.04**	**1.12 ± 0.04**	**30.59 ± 7.15**	**12.75 ± 0.59***
BPS 25 mg/kg	**26 ± 3.23**	**1.01 ± 0.07**	**0.97 ± 0.03**	**39.63 ± 8.17**	**13.77 ± 0.25**
BPS 50 mg/kg	**28 ± 3.81**	**1.01 ± 0.2**	**1.00 ± 0.09**	**28.57 ± 6.48**	**13.34 ± 0.44**

Values are expressed as mean ± SEM.

*, **, *** indicate significant difference at probability value $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control.

ANOVA followed by Dunnet's Comparison test.

Table 7. Effects of sub-chronic exposure of different concentrations of bisphenol A and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) on the biochemical parameters of rats

Treatments	Parameters			
	CAT (u/mg Protein)	**LPO (nM TBARS/ min/mg protein)**	**Total ROS (U/g tissue)**	**Protein (mg/0.5 g)**
Control	14.87 ± 0.27	13.92 ± 0.24	0.74 ± 0.01	333.91 ± 09.28
BPA 5 mg/kg	13.11 ± 0.44*	12.84 ± 0.40	0.90 ± 0.05	283.89 ± 23.59*
BPA 25 mg/kg	12.63 ± 0.38**	13.35 ± 0.32	0.77 ± 0.01	270.23 ± 08.79**
BPA 50 mg/kg	13.58 ± 0.40	15.53 ± 0.24*	1.30 ± 0.04***	280.90 ± 11.92*
BPB 5 mg/kg	14.11 ± 0.28	14.15 ± 0.51	0.95 ± 0.03	284.77 ± 04.02*
BPB 25 mg/kg	13.18 ± 0.23*	14.01 ± 0.38	0.86 ± 0.10	291.80 ± 18.68
BPB 50 mg/kg	13.89 ± 0.23	15.78 ± 0.27**	1.38 ± 0.07***	285.08 ± 04.61*
BPF 5 mg/kg	13.53 ± 0.43	14.40 ± 0.37	0.74 ± 0.07	281.28 ± 16.76*
BPF 25 mg/kg	12.86 ± 0.34**	14.64 ± 0.24	0.79 ± 0.03	264.26 ± 04.89***
BPF 50 mg/kg	12.57 ± 0.34**	15.99 ± 0.22***	1.11 ± 0.13**	288.07 ± 03.03
BPS 5 mg	12.68 ± 0.52**	14.22 ± 0.31	0.82 ± 0.04	283.61 ± 05.44*
BPS 25 mg/kg	13.66 ± 0.44	14.22 ± 0.31	0.75 ± 0.06	284.78 ± 04.83*
BPS 50 mg/kg	13.16 ± 0.58*	15.82 ± 0.24**	1.18 ± 0.08***	273.02 ± 08.89**

Values are expressed as mean ± SEM.

*, **, *** indicate significant difference at probability value $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control.

ANOVA followed by Dunnet's Comparison test.

BPA and its analogues BPB, BPF and BPS effects on the intra-testicular testosterone and plasma testosterone concentrations in rats

Plasma testosterone concentrations in different treatment groups and control are presented in table 8. Significant reduction was observed in BPA 5mg/kg ($P < 0.05$), BPA 25 mg/kg ($P < 0.01$) and BPA 50 mg/kg ($P < 0.05$) treated groups when compared to control. Testosterone concentration was reduced significantly ($P < 0.05$ and $P < 0.01$) in BPS 5 and 50 mg/kg treated groups. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose levels of 5 and 50 mg/kg. However, BPF 25 mg/kg treated group did not affect testosterone concentrations significantly in comparison to the control. On the other hand, BPS 50 mg/kg significantly reduced ($P < 0.05$) plasma testosterone concentrations; however, other doses did not reduce plasma testosterone as compared to the control.

Intra-testicular testosterone concentrations in the testis after 28 days of exposure showed significant reduction in BPA 25 and 50 mg/kg ($P <0.05$ and $P < 0.01$) as compared to the control. All doses of BPB and BPF caused significant reduction ($P < 0.01$) in intra-testicular testosterone when compared to the control. Similarly, BPS 5 and 50 mg/kg reduced ($P < 0.05$ and $P < 0.01$) intra-testicular testosterone concentration as compared to control group. Intra-testicular testosterone was not different in BPA 5 mg/kg and BPS 25 mg/kg than control as presented in table 8.

Table 8. Effects of sub-chronic exposure to different concentrations of bisphenol A and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) on the testicular testosterone concentrations in rats

Treatments	Parameters	
	Plasm Testosterone (ng/ml)	Intra-Testicular Testosterone (ng/g tissue)
Control	5.90 ± 0.18	54.27 ± 0.82
BPA 5 mg/kg	3.96 ± 0.23*	50.77 ± 2.74
BPA 25 mg/kg	3.77 ± 0.29**	45.07 ± 1.59*
BPA 50 mg/kg	3.88 ± 0.41*	41.90 ± 0.30**
BPB 5 mg/kg	3.81 ± 0.37*	42.59 ± 0.78**
BPB 25 mg/kg	4.16 ± 0.26*	44.03 ± 0.33**
BPB 50 mg/kg	3.71 ± 0.22**	42.15 ± 3.12**
BPF 5 mg/kg	3.89 ± 0.17*	43.46 ± 0.81**
BPF 25 mg/kg	4.29 ± 0.36	43.70 ± 0.55**
BPF 50 mg/kg	3.99 ± 0.14*	42.46 ± 2.26**
BPS 5 mg	4.39 ± 0.54	45.82 ± 0.68*
BPS 25 mg/kg	4.45 ± 0.31	47.65 ± 0.94
BPS 50 mg/kg	3.93 ± 0.30*	44.71 ± 0.17**

Values are expressed as mean ± SEM.

*, **, *** indicate significant difference at probability value $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control.

ANOVA followed by Dunnet's Comparison test.

Histomorphological observations after exposure of BPA and its analogues BPB, BPF and BPS

Histomorphological studies revealed changes in area of seminiferous tubules and interstitium, seminiferous tubules diameter and epithelial height after 28 days of exposure are presented in table 9 and fig 8. There was no significant difference observed in the area of seminiferous tubule % and area of interstitium % of different treatment groups as compared to control. Similarly, significant difference was also not observed in diameter of seminiferous tubule in all treated groups as compared to control group. On the other hand, BPA 50 mg/kg significantly reduced ($P < 0.05$) epithelial height. Significant reduction ($P < 0.01$) in the epithelial height was also observed in BPB, BPF 50 mg/kg as compared to the control group. Similarly, BPS 50 mg/kg group reduced ($P < 0.05$) epithelial height as compared to control. Epithelial height was not different in BPA, BPB, BPF and BPS 5, 25 mg/kg than control.

In the control group testis with thick epithelium, sperm filled lumen and seminiferous tubules were observed in the fig 8. Seminiferous tubules arrangement and shape was not very different in all treated groups when compared to the control. Though, the pattern of epithelium was thin and the number of secondary spermatocytes was reduced in the treated groups when compared to the control. The groups with higher dose (50 mg/kg day) were observed to have very few tubules and there were no elongated spermatids in the lumen when these were compared to control (fig 8).

Histomorphological observations in the testis and epididymis after exposure to BPA, BPB, BPF and BPS

Morphometry of different parameters of epididymal caput and cauda region after 28 days of sub chronic exposure did not show any significant difference in any of the parameter (Tubular and lumen diameter, epithelial height and percentage of epithelium and lumen) as compared to the control presented in table 9 and 10. The shape of cauda and caput of the epididymis in the control was not very different of that of the treated groups presented in the fig 9 and 10. In the groups treated with 25 and 50 mg/kg day very few empty lumen observed in each epididymal section when compared to the control though there was no loss of sterocilia observed in these groups.

The number of different cell types in the seminiferous tubules is presented in table no 10. Significant difference was not observed in any of the treated groups of BPA and its analogues BPB, BPF and BPS as compared to the control group. Though, the number of cells like spermatids and spermatocytes had decreased in some of the treated groups when compared to the control but that reduction was not statistically different when compared to the control.

Table 9. Effects of sub-chronic exposure to different concentrations of bisphenol A and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) on the testis morphometry

Treatments	Parameters			
	Area of seminiferous tubule (%)	**Area of Intrstitium (%)**	**Seminiferous tubule diameter (µm)**	**Epithelial height**
Control	85.64 ± 1.89	15.87 ± 1.15	207.62 ± 1.79	71.48 ± 1.92
BPA 5 mg/kg	83.83 ± 1.31	16.06 ± 1.47	201.30 ± 3.16	69.05 ± 1.03
BPA 25 mg/kg	82.96 ± 1.15	16.56 ± 1.21	205.38 ± 1.57	65.55 ± 1.30
BPA 50 mg/kg	81.70 ± 1.64	17.28 ± 1.33	204.06 ± 1.50	59.38 ± 2.20*
BPB 5 mg/kg	83.92 ± 1.61	16.97 ± 1.22	205.48 ± 1.62	68.89 ± 1.30
BPB 25 mg/kg	82.66 ± 1.22	16.50 ± 1.48	205.68 ± 1.48	69.21 ± 1.32
BPB 50 mg/kg	83.78 ± 1.25	16.80 ± 1.39	204.10 ± 1.27	58.04± 2.75**
BPF 5 mg/kg	84.53 ± 1.39	16.62 ± 1.57	204.44 ± 1.61	69.22 ± 2.13
BPF 25 mg/kg	83.64 ± 1.46	16.69 ± 1.46	202.53 ± 1.74	66.52 ± 1.77
BPF 50 mg/kg	83.79 ± 1.36	17.40 ± 1.42	204.28 ± 1.23	59.20± 2.54**
BPS 5 mg	84.50 ± 1.31	17.70 ± 1.47	203.84 ± 1.26	69.03 ± 2.68
BPS 25 mg/kg	83.68 ± 1.44	17.68 ± 1.51	204.48 ± 1.59	64.22 ± 1.89
BPS 50 mg/kg	84.36 ± 1.34	16.57 ± 1.75	203.66 ± 1.46	58.64 ± 2.75*

Values are expressed as mean ± SEM.

*, ** indicate significant difference at probability value $P < 0.05$ and $P < 0.01$ compared to control.

ANOVA followed by Dunnet's Comparison test.

Table 10. Effects of sub-chronic exposure to different concentrations of bisphenol A and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) on the epididymal caput morphometry

Treatments	Parameters				
	Tubular diameter (µm)	Lumen diameter (u)	Epithelial height (µm)	Epithelium (% age)	Lumen (% age)
Control	402.48 ± 5.72	295.82 ± 5.55	30.42 ± 1.05	36.62 ± 1.51	65.58 ± 1.78
BPA 5 mg/kg	398.55 ± 6.91	286.00 ± 9.91	29.51 ± 0.97	34.39 ± 1.20	63.42 ± 1.51
BPA 25 mg/kg	390.90 ± 7.57	276.51 ± 7.34	25.98 ± 1.10	32.55 ± 1.06	62.80 ± 1.28
BPA 50 mg/kg	389.63 ± 10.31	283.03 ± 12.5	26.84 ±1.17	33.55 ± 1.47	63.94 ± 1.27
BPB 5 mg/kg	395.13 ± 9.82	293.19 ± 9.10	28.98 ± 0.66	32.16 ± 1.56	63.61 ± 1.12
BPB 25 mg/kg	393.53 ± 7.16	287.48 ± 9.21	27.88 ± 0.90	33.50 ± 1.48	63.93 ± 1.34
BPB 50 mg/kg	391.75 ± 8.99	289.80 ± 7.17	28.82 ± 1.30	30.67 ± 1.32	63.40 ± 1.26
BPF 5 mg/kg	389.69 ± 7.01	291.11 ± 8.81	27.63 ± 0.94	29.23 ± 1.02	62.40 ± 1.39
BPF 25 mg/kg	391.28 ± 10.69	286.98 ± 6.56	29.35 ± 1.35	32.61 ± 1.12	62.40 ± 1.48
BPF 50 mg/kg	391.26 ± 8.76	288.65 ± 10.6	27.19 ± 0.98	33.51 ± 1.35	62.35 ± 1.56
BPS 5 mg	394.13 ± 8.29	289.34 ± 8.26	27.80 ± 1.11	34.75 ± 1..39	63.77 ± 1.27
BPS 25 mg/kg	390.13 ± 8.19	279.21 ± 10.3	29.00 ± 1.15	32.45 ± 1.41	63.36 ± 1.37
BPS 50 mg/kg	391.30 ± 10.13	284.51 ± 11.4	27.40 ± 1.20	34.25 ± 1.50	62.22 ± 1.78

Values are expressed as mean ± SEM.

ANOVA followed by Dunnet's Comparison test.

Table 11. Effects of sub-chronic exposure to different concentrations of bisphenol A and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) on the epididymal cauda morphometry

Treatments	Parameters				
	Tubular diameter (µm)	**Lumen daimeter (u)**	**Epithelial height (µm)**	**Epithelium (% age)**	**Lumen (% age)**
Control	481.73 ± 8.84	428.13 ± 11.14	28.98 ± 1.58	35.88 ± 1.92	61.78 ± 2.11
BPA 5 mg/kg	474.46 ± 12.55	429.21 ± 10.10	32.40 ± 1.70	38.65 ± 2.11	57.18 ± 2.20
BPA 25 mg/kg	473.80 ± 11.46	435.46 ± 9.09	29.55 ± 1.47	36.27 ± 1.89	61.46 ± 1.99
BPA 50 mg/kg	477.19 ± 10.68	431.92 ± 9.52	31.11 ± 1.65	36.68 ± 1.95	59.53 ± 1.98
BPB 5 mg/kg	470.11 ± 10.53	432.46 ± 9.77	30.32 ± 1.43	36.68 ± 1.91	62.86 ± 1.91
BPB 25 mg/kg	479.50 ± 10.12	429.76 ± 11.62	28.63 ± 1.58	37.53 ± 1.90	61.52 ± 1.96
BPB 50 mg/kg	477.86 ± 12.45	429.90 ± 9.36	31.55 ± 1.62	38.93 ± 1.95	59.11 ± 2.06
BPF 5 mg/kg	477.32 ± 10.51	435.46 ± 9.09	28.73 ± 1.48	36.01 ± 1.91	62.93 ± 1.99
BPF 25 mg/kg	477.19 ± 10.68	427.76 ± 10.52	31.11 ± 1.65	36.39 ± 1.96	62.95 ± 1.95
BPF 50 mg/kg	477.25 ± 9.92	422.73 ± 9.90	29.53 ± 1.53	36.39 ± 1.96	63.60 ± 1.96
BPS 5 mg/kg	473.80 ± 11.46	435.46 ± 9.09	29.15 ± 1.40	35.91 ± 1.91	62.58 ± 1.83
BPS 25 mg/kg	482.78 ± 9.61	434.63 ± 9.22	29.34 ± 1.44	36.39 ± 1.96	62.45 ± 2.02
BPS 50 mg/kg	479.28 ± 7.92	434.34 ± 9.44	29.53 ± 1.53	36.39 ± 1.96	63.60 ± 1.96

Values are expressed as mean **±** SEM.

ANOVA followed by Dunnet's Comparison test.

Table 12. Effects of sub-chronic exposure to different concentrations of bisphenol A and its analogues BPB, BPF and BPS (5, 25 and 50 mg/kg) in rats seminiferous tubules and number of different types of cells

Treatments	Parameters		
	Spermatogonia	**Spermatocytes**	**Spermatids**
Control	62.08 ± 0.79	77.10 ± 1.06	250.26 ± 1.97
BPA 5 mg/kg	61.26 ± 0.82	75.40 ± 1.29	247.36 ± 2.45
BPA 25 mg/kg	59.72 ± 0.87	73.32 ± 0.97	248.48 ± 2.61
BPA 50 mg/kg	59.84 ± 0.66	72.18 ± 1.20	245.32 ± 2.06
BPB 5 mg/kg	59.76 ± 0.60	74.32 ± 0.94	246.74 ± 1.84
BPB 25 mg/kg	58.92 ± 0.69	73.54 ± 1.42	245.98 ± 2.24
BPB 50 mg/kg	59.08 ± 0.88	71.82 ± 1.29	244.72 ± 2.26
BPF 5 mg/kg	58.00 ± 0.97	73.64 ± 1.35	247.62 ± 2.20
BPF 25 mg/kg	58.94 ± 1.21	72.64 ± 1.24	247.44 ± 2.43
BPF 50 mg/kg	59.54 ± 0.75	71.50 ± 1.26	245.16 ± 1.97
BPS 5 mg	60.64 ± 1.00	74.74 ± 1.30	247.42 ± 2.46
BPS 25 mg/kg	59.88 ± 1.24	73.84 ± 1.23	246.70 ± 2.36
BPS 50 mg/kg	60.48 ± 0.89	72.12 ± 1.24	245.02 ± 2.10

Values are expressed as mean ± SEM.

ANOVA followed by Dunnet's Comparison test.

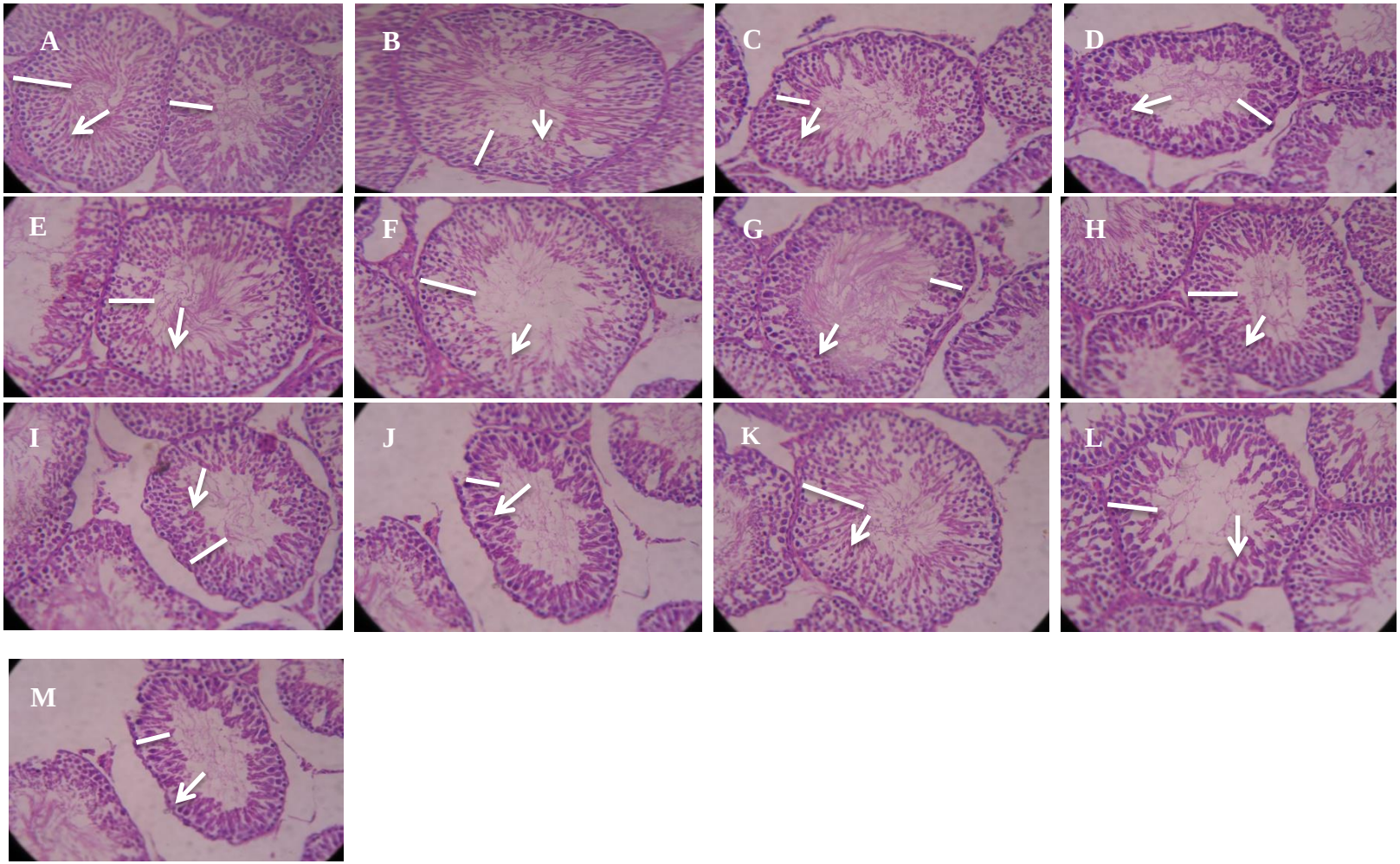

Fig. 8. Photomicrograph from testicular tissue showing (A) control; with compact arrangement of seminiferous tubules with thick epithelial height (Line without arrow head) and elongated spermatids (arrow) (B, C and D); BPA (5, 25 and 50 mg/kg/day) treated groups presenting seminiferous tubules with epithelium (line without arrow head) and spermatids (white arrow): (E, F and G) BPB (5,25 and 50 mg/kg/day) treated groups presenting seminiferous tubules with epithelium (line without arrow head) and elongated spermatids (white arrow) (H, I and J) BPF (5, 25 and 50 mg/kg/day) treated groups presenting seminiferous tubules with epithelium (line without arrow head) and elongated spermatids (white arrow); (K, L and M) BPS (5, 25 and 50 mg/kg/day) treated groups presenting seminiferous tubules with epithelium (line without arrow head) and spermatids (white arrow). H&E (x40)

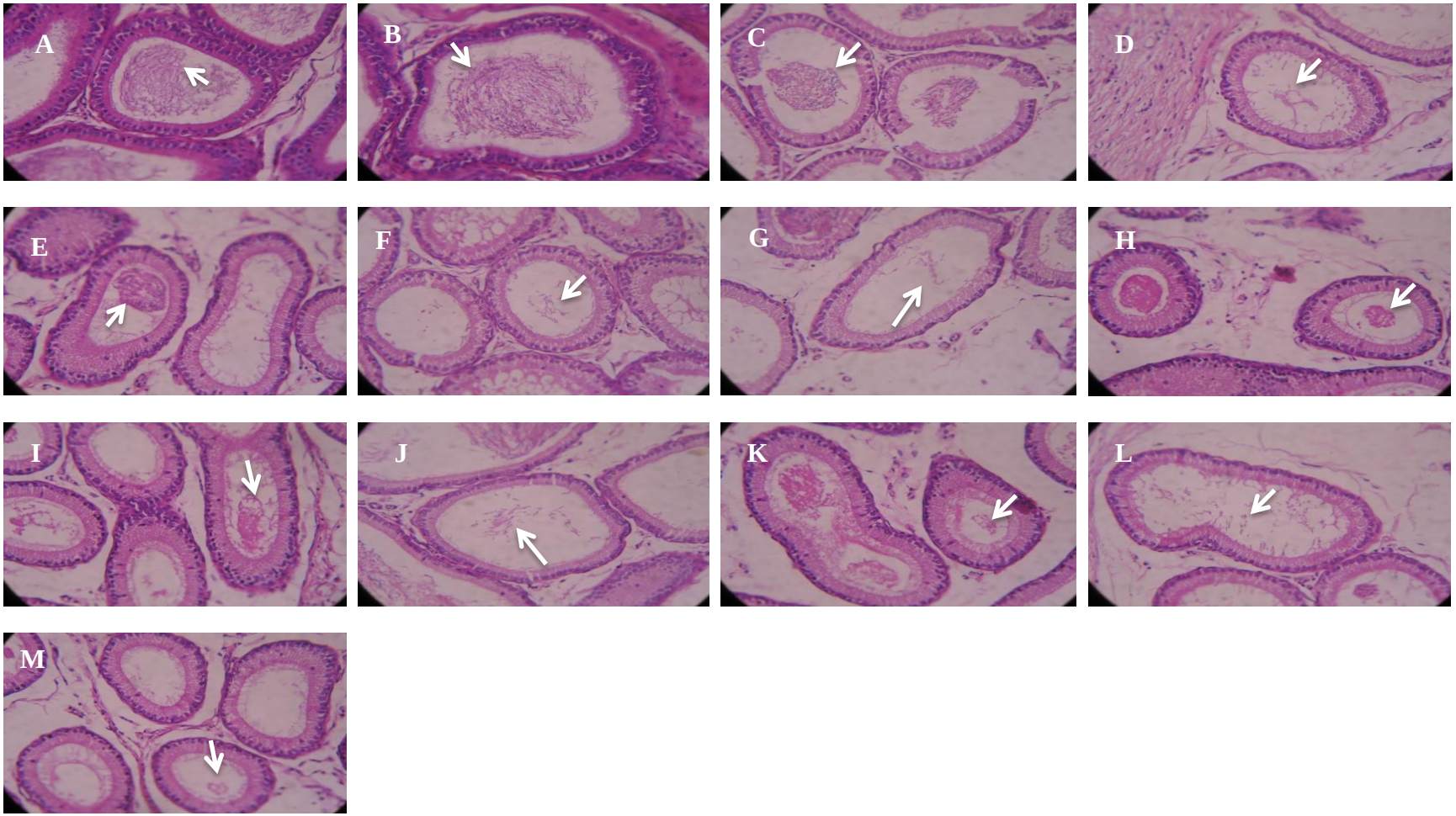

Fig. 9. Photomicrograph of cross section of caput epididymis from: (A) Control; showing normal morphology with compactly arranged tubules and thick epithelium, lumen filled with sperm (arrow), (B, C and D) BPA (5, 25, 50 mg/kg/day) treated groups presenting tubules with epithelium and lumen with sperms (white arrow), (E, F and G) BPB (5, 25 and 50 mg/kg/day) treated presenting caput tubules with epithelium and sperm in the lumen, (H, I and J) BPF (5, 25, 50 mg/kg/day) treated groups presenting tubules with epithelium and sperm in the lumen, (K, L and M) BPS (5, 25 and 50 mg/kg/day) treated groups presenting tubules with epithelium and sperm in the lumen (white arrow).H&E (x40)

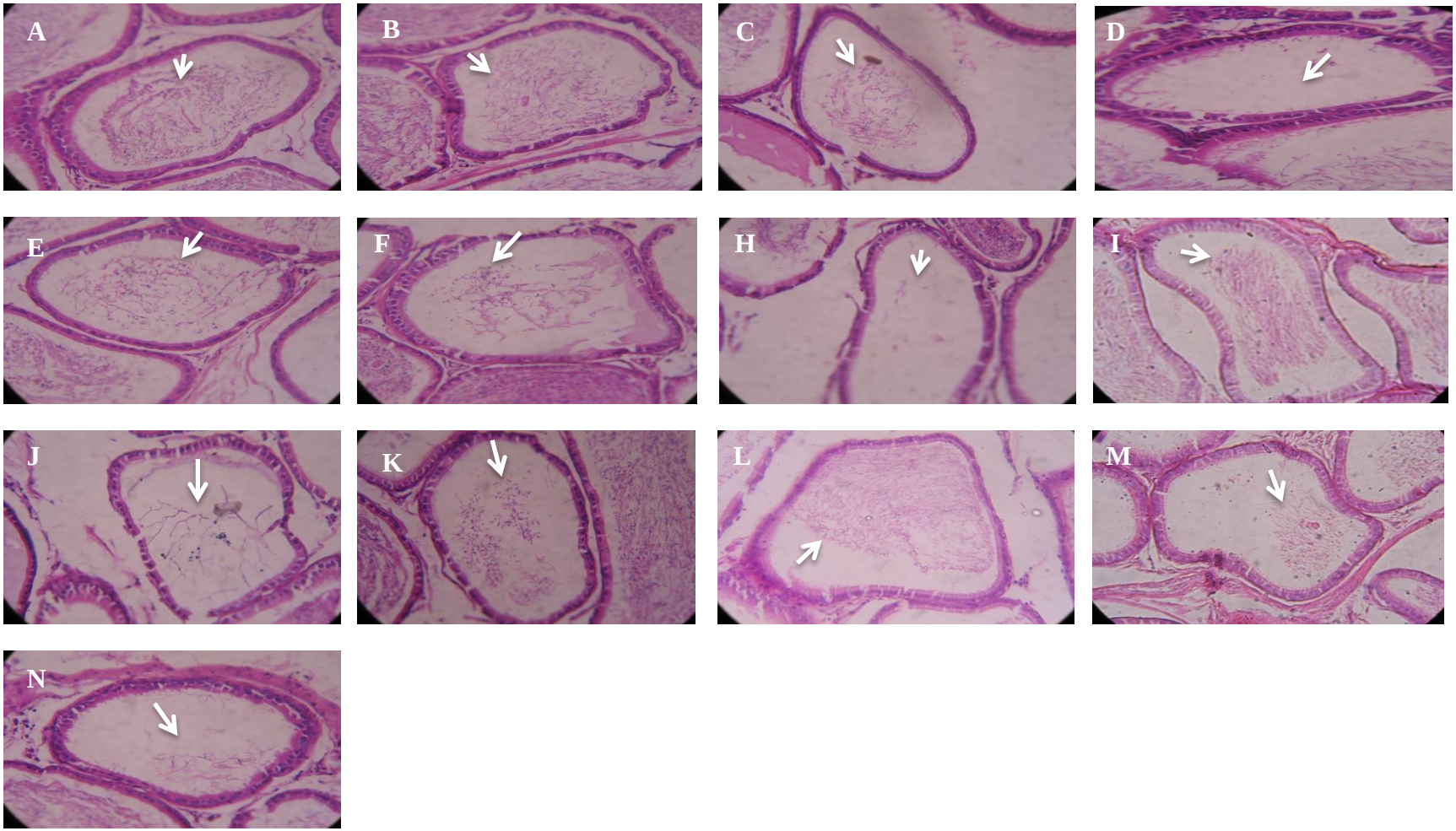

Fig. 10. Photomicrograph of cross section of cauda epididymis from: (A) Control; showing normal morphology of cauda epididymis with tubules and thick epithelium, lumen filled with sperms (arrow), (B, C and D) BPA (5, 25, 50 mg/kg/day) treated groups presenting cauda tubules with epithelium and sperms in the lumen (white arrow): (E, F and G) BPB (5, 25 and 50 mg/kg/day) treated groups presenting cauda tubules with sperm in the lumen (white arrow). (H, I and J) BPF (5, 25 and 50 mg/kg/day) treated groups presenting cauda tubules with sperm in the lumen. (K, L and M) BPS (5, 25 and 50 mg/kg/day) treated presenting cauda tubules with sperm in the lumen. (white arrow).H&E (x40)

DISCUSSION

Restriction on use of BPA in the market has led in a way to the use of its alternatives as BPB, BPF and BPS, which are also reported unsafe. It is thought that the production of these analogues is going to increase in future due to the ban on BPA use in several countries of the world. It is not only concerning but also alarming that these analogues have been reported in several edible samples which increase the threat of both general and occupationally exposed people. The similarity in its structure with BPA, these analogues can act like endocrine disrupters (Usman and Ahmad, 2016). *In vitro* data provided in mammals, suggest that both BPF and BPS are capable of binding many receptors to change testosterone secretions in fetal testis assay and can induce cell proliferation (Kitamura *et al.*, 2005, Molina-Molina *et al.*, 2013, Delfosse *et al.*, 2012, Eladak *et al.*, 2015). There is very little data so far available using *in vivo* studies in mammalian and non-mammalian models but some studies have shown that these compounds have impact on the expression of hormone regulated genes and can exhibit reproductive and developmental effects (Ji *et al.*, 2013, Kinch *et al.*, 2015, Naderi *et al.*, 2014, Cano-Nicolau *et al.*, 2016b). The present data is raising concern that whether the so called exposure to safer alternatives to BPA is either safe or more threatening to living organisms. Very limited data is available on the analogues of BPA which can confirm whether they are really safe or it is just a commercial shift in order to continue the BPA family in the market. We conducted two experiments both *in vivo* to show the toxic effect of BPA and its analogues on testicular tissues and reproductive function in male rats, based on current literature, BPS, BPF and BPB have already been detected in consumer products as alternatives for BPA (Rochester and Bolden, 2015).

In the *in vivo* study, we aimed to assess the effects of BPA and its commonly used analogues, BPB, BPF and BPS on the antioxidant status of testicular tissues. Antioxidant enzymes like CAT and SOD play vital role in mechanism against oxidative stress in the body (Pandey and Rizvi, 2010). ROS formation is adversely effected by many toxicants which damage cellular network and structure. Toxic influence of phenols is associated with ROS suggested by many researchers like (Michałowicz and Duda, 2007) who showed that BPA plays an important role in the formation of ROS and oxidation of many cellular biomolecules in the body (Zhan *et al.*, 2006). Though, the values of ROS and POD in our study were significantly different as compared to the

control groups which are similar to the *in vitro* effect of BPS observed by (Ullah *et al.*, 2016). BPA and its analogues have also been observed to increase the levels of ROS in human peripheral blood mononuclear cells (BBMCs) (Michałowicz, 2014). A study by (Maćczak *et al.*, 2017) showed that the mechanism of oxidative action of BPA and its analogues, BPB, PBF and BPAF increased the level of ROS, caused lipid peroxidation and also altered the activates of SOD and CAT in mature erythrocytes. In the *in-vivo* study we observed that there was dose dependent effect of BPA and its analogues BPB, BPF and BPS on the oxidative stress in reproductive system of rats. In the higher doses tested groups we observed that there was significant change in the histology of the reproductive tissues by reducing the number of sperms in the lumen of epididymis and decreasing the height of epithelial tissues of seminiferous tubules. This is not surprising as estrogen, while essential for normal epididymis function, has inhibitory effects on the brain, pituitary and gonadal axis in males, and it is well documented that elevated E2 inhibits spermatogenesis and testicular testosterone secretion (Richter *et al.*, 2007). Interference with androgen action during gonadal development can also cause abnormalities of the male reproductive system (Lee *et al.*, 2003). High doses also induced higher oxidative stress in the tissues as compared to the low dose groups and control. The above changes can be because of increase in ROS (Devasagayam *et al.*, 2004). ROS, which is produced in the mitochondria produces free oxygen ions during normal metabolism which help in homeostasis and cell signaling (Rejitha and Karthiayini, 2013). If this level of ROS continues in the same rate it results in DNA damage and damage to lipids and protein. In order to overcome this situation, the cell activates its antioxidant enzymes production which is the self-defense mechanism of the body which help in reducing the levels of ROS (Kaul and Forman, 2000). When cells are unable to detoxify ROS they go into oxidative stress which causes reduction in the level of antioxidants (Kaul and Forman, 2000, Pérez *et al.*, 2009).

In our study results it seem that BPA and its analogues BPB, BPF and BPS levels caused the induction of ROS which led to surge in LPO levels and activation of antioxidants of the tissues which are in line with the earlier study where the degradation of protein in cell occurred due to bisphenol exposure (Michałowicz *et al.*, 2015). In some of the other studies it is also found that if this oxidative stress persists it can cause injury to the cell membranes known as LPO (Mokra *et al.*, 2015, Lee *et al.*, 2013, Feng *et al.*, 2012). BPA and BPS also caused protein and DNA damage in cell in an *in-vitro* studies (Rotroff *et al.*, 2013). Oxidative stress was also observed in

the *in vivo* studies where the levels of ROS and LPO increased to an observable level. There was also change observed in the SOD and CAT of different treated groups which also indicates oxidative stress in the tissue. These high levels of ROS and LPO also indicate that this change occurred because of the oxidative stress caused by BPA and its analogues BPB, BPF and BPS which reduced the level of antioxidants and protein in the tissues as described by (Radák *et al.*, 1999).

Testosterone concentrations in the *in vivo* study showed significant change when matched to the control group. There was substantial change noted in both intra-testicular and plasma testosterone concentration in the treated groups of *in vivo* study as compared to the control group. Both intra-testicular and plasma testosterone levels reduced in the treated groups in comparison with the control group. Rosenmai in 2014 investigated the effects of BPA alternatives BPF and BPS on steroidogenesis and observed that BPA and its analogues BPF and BPS altered the steroidogenesis pathway as noticed in our study (García *et al.*, 2012, Rosenmai *et al.*, 2014).

Reproductive hormones and cellular interactions in the testes control the process of spermatogenesis. Disturbed antioxidant enzymes because of ROS lead into altered spermatogenesis. In the present study the higher ROS levels have altered the levels of androgens. These altered levels of androgens lead into less number of spermatids, thin epithelial height and seminiferous tubules in the testicular tissues and reduce concentrations of testosterone in the control group when compared to the treated groups. In the earlier studies it was observed that BPA and BPS, exposure alter steroidogenesis, reduce gene transcripts for GnRH and oxidative stress in the different tissues (Ullah *et al.*, 2016, Allard, 2014, Manfo *et al.*, 2014, Jambor *et al.*, 2017, Ji *et al.*, 2013, Lee *et al.*, 2018). Prominently our present study shows that BPA and its analogues BPB, BPF and BPS act as inducers for the oxidative stress which alters spermatogenesis in the testis by reducing the testosterone secretion. In this context both *in-vivo* and *in-vitro* specific mechanism based studies are needed to determine the GnRh transcripts which may show the cell and tissue specific response in the environment hazard assessment of these substitutes of BPA and EDCs which will also highlight the molecular mechanism in understanding the comparison of *in-vitro* and *in-vivo* studies.

CONCLUSIONS

Findings of our present investigations suggest that BPA and its analogues BPB, BPF and BPS not only show anti-androgenic properties but also lead into oxidative stress which causes disturbances in the reproductive function of adults rats. However, in order to understand the exact mechanism of these conditions, different studies need to be carried out both *in vivo* and *in vitro* with different low and high doses of these all analogues of BPA to understand the biochemical, physiological and endocrine effects in different animals.

ABSTRACT

Background: Bisphenol A (BPA), an estrogen mimicking endocrine disrupting chemical also known as an environmental contaminant used for the manufacturing of polycarbonate plastics and epoxy resins with toxic effects on male reproductive system. Due to its well ascertained toxicity as endocrine disruptor, industries have started to replace it with BPA analogues whose alleged greater safety is scarcely supported by literature studies.

Materials and Methods: In the present study we investigated whether the chronic exposure to low BPA dose affects spermatogenesis through oxidative stress on the male reproductive system. To evaluate the influence of chronic exposure of BPA and its analogues BPB, BPF and BPS adult healthy male rats (22 days old) were exposed to water containing different concentrations of bisphenols (5, 25 and 50 μg/L) in drinking water for duration of 48 weeks. After the completion of the experimental period, animals were dissected and different parameters (hormone concentrations, histology of testis and epididymis, oxidative stress in the testis, and sperm parameters) were determined.

Results: Results of the present study showed significant alterations in the gonadosomatic index (GSI) and relative reproductive organs weights after the treatment with BPA and its analogues BPB, BPF and BPS. Oxidative stress in the testis was significantly elevated while sperm motility, daily sperm production (DSP) and number of sperm in epididymis were reduced. Plasma testosterone, Leutenizing hormone (LH) and Follicle Stimulating hormone (FSH) concentrations were reduced and estradiol levels were high in 50 μg/L exposed groups as compared to control. Histological observations showed significant reduction in the epithelial height of the testis along with disrupted spermatogenesis. Other prominent observations were empty lumen of the seminiferous tubules and caput region of the epididymis after exposure to BPA and its analogues BPB, BPF and BPS.

Conclusions: These results suggest that exposure to BPA and its analogues BPB, BPF and BPS for long duration can induce structural changes in testicular tissue and endocrine alterations in the male reproductive system.

INTRODUCTION

Plasticizer such as bisphenol A (BPA) is an environmental pollutant detected in wildlife, humans samples and environment (Corrales *et al.*, 2015). BPA exposure is associated with many human diseases and is suspected to affect many body's physiological functions (Chevalier and Fénichel, 2015, Seachrist *et al.*, 2016, Chen *et al.*, 2016a). Having several concerns for a safer world of BPA there have been several alternatives of BPA introduced into environment known as BPA analogs (Chen *et al.*, 2016a). Bisphenol B (BPB), bisphenol F (BPF) and bisphenol S (BPS) have been introduced into the environment as BPA alternative which are used for the production of Plastics, epoxy resins, polycarbonates for lining large food containers, water pipes and coatings of Food containers, dyes, paper products and food packaging materials (Rochester and Bolden, 2015, Chen *et al.*, 2016a, Eladak *et al.*, 2015, Goodson *et al.*, 2002, Yang *et al.*, 2014a, Kinch *et al.*, 2015, Danzl *et al.*, 2009). BPA analogues have increased concern regarding emerging environmental pollutants where some of these analogues are detected in concentrations higher than BPA (Chen *et al.*, 2016a, Caballero-Casero *et al.*, 2016). For example, in a study from Italy the concentrations of BPB were higher than BPA in serum samples of healthy women and endometriotic women (Caballero-Casero *et al.*, 2016). similarly, in another study from Saudi Arabia in the urine of general population the concentrations of both BPS and BPF were higher than BPA (Chen *et al.*, 2016a). In another study food products sold in New York and Albany were analyzed and 75% were detected with BPA and its analogues measurable amounts (Liao and Kannan, 2013). BPS and BPF have been identified up to detectable amounts in food items and paper products (Liao and Kannan, 2014b, Goldinger *et al.*, 2015, Russo *et al.*, 2017). Across the Globe several studies have shown detectable amounts of BPA analogs in the urinary samples, umbilical cord samples and maternal samples (Asimakopoulos *et al.*, 2016, Heffernan *et al.*, 2016, Ye *et al.*, 2015, Lu *et al.*, 2016, Liu *et al.*, 2017). BPA and its analogs observed in *in vitro* studies induced a number of physiological changes in cell lines of red blood cells, preadipocytes and testis (Boucher *et al.*, 2016b, Desdoits-Lethimonier *et al.*, 2017, Maćczak *et al.*, 2017, Mokra *et al.*, 2017). Studies on rodents showed that BPA analogues affect hormone concentrations, testis function, sperm production and sperm DNA damage (Li *et al.*, 2016, Shi *et al.*, 2017, Oliveira *et al.*, 2017, Castro *et al.*, 2013). Many studies of BPA and its anaogues

suggest that these chemicals have greater neuroendocrine disruptive effects as BPA where they lead to complex behavioral changes in rodent species (Kim *et al.*, 2015, Ohtani *et al.*, 2017, Catanese and Vandenberg, 2016, Rosenfeld, 2017). Where, these chemicals also affect the gene expression in hypothalamus and other brain areas (Cano-Nicolau *et al.*, 2016a, Qiu *et al.*, 2015, Zhang *et al.*, 2017a, Zhang *et al.*, 2018a, Qiu *et al.*, 2018a, Huang *et al.*, 2016). BPA analogs have also been studied to induce hormonal imbalance in E2 synthesis, thyroid hormone production and testosterone levels (Cano-Nicolau *et al.*, 2016a, Li *et al.*, 2016, Le Fol *et al.*, 2017, Kwon *et al.*, 2016).

In vitro and *in vivo* studies regarding BPA analogues are scare and limited data has shown that these chemicals have reproductive toxicity (Chen *et al.*, 2016a, Naderi *et al.*, 2014). These chemicals are estrogenic in nature and have endocrine disrupting potentials (Yamasaki *et al.*, 2004, Rosenmai *et al.*, 2014, Kitamura *et al.*, 2005). BPB, BPF and BPS are considered as safe alternatives to BPA and it is important to understand that whether actions of these compounds are similar or ahve more potent endocrine disruptor than BPA.

In summary the current study provides information about the so called safer alternatives to BPA which have shown similar endocrine disturbances as BPA in animal studies. Most of these disturbances are associated with change in steroid or alterations at the non-steroid pathways. In current study we reported that low concentration of these compounds for a long period can impair spermatogenic output and hence change the normal process of spermatogenesis in rats. Changes in the process of steroidogenesis suggest that chronic exposure to BPA and its analogues BPB, BPF and BPS have endocrine disrupting properties by affecting the male reproductive functions in Sprague Dawley rats.

MATERIALS AND METHODS

Animals

Male healthy rats (n = 91), weighing (30 – 40 g) were separated from their mothers on postnatal day 22 (PND 22) and were randomly divided into thirteen groups. Animals were kept in steel cages (7 animals/cage) at temperature 22-25 ^{o}C and controlled light and dark cycle of 14 – 10 hrs light/dark was maintained throughout the experimental period. Animals were fed with laboratory feed (soy and alfalfa free) and water in poly sulfone bottles was available at *ad libitum*. All the experimental protocols were approved by the ethical committee of the department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

Experimental design

From PND 23, animals (n=91) were allocated into thirteen different groups. First group served as control and was provided with water containing (0.1% ethanol), while 2nd, 3rd and 4th groups were served with water containing 5, 25 and 50 µg/L of BPA respectively. While 5th, 6th and 7th groups were served with water containing 5, 25 and 50 µg/L of BPB. Similarly, 8th, 9th and 10th groups received water containing 5, 25 and 50 µg/L of BPF and BPS was also given in water to 11th, 12th and 13th groups at a concentration of 5, 25 and 50 µg/L. Bisphenols dissolved in ethanol and the stock solutions were diluted with water (final concentration of ethanol in the water was kept below 0.1%). Animals were provided with water alone or water with different concentrations of BPA, BPB, BPF and BPS for the period of 48 weeks. The duration of the exposure was selected according to the OECD test guideline 452 and the doses were selected on the basis of previous studies by (Ji *et al.*, 2013)and (Chen *et al.*, 2017). The BPA, BPB, BPF and BPS solutions in the water bottles was daily replaced with fresh solutions.

After the completion of the experimental period, animals were weighed, and seven animals per group were euthanized by cervical dislocation. Blood was collected from heart through cardiac puncture in heparinized syringes and was subjected to centrifugation at 3000 rpm for 15 min. Plasma was isolated and kept at −20 °C for hormonal assay. Reproductive organs (testis, epididymis, seminal vesicle and prostate) were dissected out and weighed for calculation of gonadosomatic index (GSI) and relative organs weight. Right epididymis and right testis were

used for histology while left testis was used for DSP and biochemical analysis. Left epididymis was used for determination of sperm viability, motility and sperm count in the epididymis.

Gondosomatic index (GSI) and relative weight of organs

GSI which is an important parameter was obtained for each animal according to the formula used by Barber and Blake (Barber and Blake, 2006).

$$GSI = Gonadal\ weight\ (g) / organs\ weight\ (g) \times 100$$

Relative weight of the organs was determined according to the following formula

$$Relative\ weight\ (mg/g) = Organ\ weight\ (mg) / Body\ weight\ (g)$$

Relative weights of the organs were expressed as mg/g body weight.

Biochemical assays

Tissues were collected and were processed for the antioxidant enzymes. Tissues were homogenized with automatic homogenizer in phosphate buffer saline and centrifuged at 3,000 rpm for 30 mins. After the centrifugation the supernatant was removed and used for the hormonal analysis, protein estimation and antioxidant enzymes. Antioxidant enzymes were performed for the reproductive tissues as explained in chapter 2.

Sperm motility and viability

Immediately after dissection, the cauda epididymis was cut slightly with a scissor in 0.5 mL pre-warmed (at 37 ºC) phosphate buffered saline (pH 7.3) containing a drop of nigrosine stain. An aliquot of 50 µL was taken, placed on a pre-cleaned and warmed (at 37 ºC) glass slide and was observed under a light microscope at 40X. A total of 100 sperm/sample were analyzed for motility by a technician blinded to the treatment groups. Each sample was analyzed three times and the average value was used as the total sperm motility. For viability, a drop of eosin and nigrosine was added to the sperm sample. A volume of 10 µL was placed on a pre-warmed and cleaned glass slide and observed under a microscope at 100 X. Ten fields were analyzed by a person blinded to the treatment groups of BPA and its analogues BPB, BPF and BPS. A total of 100 sperm/field were checked for eosin staining and numbers of live and dead sperm were estimated. Each sample was repeated three times and average number was reported and expressed as percentage of live sperm.

Histopathology parameters

Reproductive tissues dissected from animals were further processed according to the histological method explained in chapter no 2 of the current study.

DSP, Number of sperm in different parts of epididymis

The different sperm parameters as (DSP and number of sperm) in the epididymis were performed according to the procedures explained in chapter no 2.

Hormonal analysis

The different reproductive hormones were determined by ELISA purchased from Amgenix Inc. USA and were performed with instructions given by the company as explained in chapter no 2.

Statistical analysis

Dunnet's multiple comparison tests which followed ANOVA was used for the comparison of different groups with control using Graph Pad Prism software. Values were expressed as Mean ± SEM and were considered significant at $P < 0.05$.

RESULTS

Effects of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 µg/L) on Initial and final body weight and body weight gain of male rats

Initial and final body weight and body weight gain of the control animals and exposed group of different concentrations of BPA and its analogues BPB, BPF, BPS is presented in table 13. At the start of the experiment all the animals were approximately of the same body weight, however, at the completion of the experiment the body weight of 50 ug/L BPA and its analogues BPB, BPF and BPS exposed groups were significantly high ($P < 0.05$) than control. On the other hand, there was no significant difference observed in the final body weight of other treated groups when compared to the control. However, the body weight gain was also comparable to the control at the end of the 48th week of treatment (Table 13).

Effects of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 µg/L) on final body weight, GSI and absolute and relative weights of reproductive organs of male rats

Absolute and relative reproductive organs weight, GSI and body weight is represented in the table 14. Significant increase ($P < 0.05$) was observed in BPA, BPB, BPF and BPS (50 ug/L) when compared to the control. While, there was no significant difference in the other treatment groups observed when compared to the control. There was no significant difference observed in paired testis when compared to the control after 48 weeks of exposure to different concentration of BPA and its analogues BPB, BPF and BPS. GSI showed significant ($P < 0.05$) reduction in BPA, BPB, BPF and BPS 50 ug/L exposed groups as compared to the control. While there was no difference observed in the other treated groups when compared to control. There was also no significant difference observed in absolute paired testis of all the treated groups of BPA and its analogues BPB, BPF and BPS when compared to the control, however, relative epididymis weight reduced significantly ($P < 0.01$) in BPA, BPB, BPF and BPS 50 ug/L treated groups. On the other hand, there was difference observed in the other treatment groups but that was not significant to the control (Table 14).

Table 13: Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 µg/L) on body weight gain of rats

Treatments	Parameters		
	Initial Body weight (g)	**Final Body Weight (g)**	**Body weight gain**
Control	30.63 ± 0.38	541.11 ± 2.02	510.37 ± 2.25
BPA 5 ug/L	32.01 ± 0.31	537.81 ± 1.24	505.81 ± 0.96
BPA 25 ug/L	31.41 ± 0.50	538.40 ± 0.40	507.11 ± 0.44
BPA 50 ug/L	32.41 ± 0.40	549.40 ± 2.65 *	517.11 ± 2.30
BPB 5 ug/L	31.98 ± 0.54	535.10 ± 1.44	503.01 ± 1.66
BPB 25 ug/L	31.41 ± 0.74	537.60 ± 1.02	506.21 ± 1.68
BPB 50 ug/L	32.61 ± 0.75	548.60 ± 1.83 *	516.11 ± 2.09
BPF 5 ug/L	31.83 ± 0.95	537.80 ± 1.24	505.97 ± 1.12
BPF 25 ug/L	32.54 ± 0.86	538.40 ± 0.40	508.46 ± 1.20
BPF 50 ug/L	32.61 ± 0.67	548.20 ± 2.69 *	515.61 ± 2.74
BPS 5 ug/L	32.61 ± 0.92	540.20 ± 2.35	506.41 ± 1.83
BPS 25ug/L	33.03 ± 0.94	538.60 ± 0.50	507.77 ± 1.01
BPS 50 ug/L	33.26 ± 0.93	548.80 ± 2.28 *	515.53 ± 2.98

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

Effects of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 µg/L) on absolute seminal vesical weight, relative seminal vesical weight, absolute prostate weight and relative prostate weight of male rats

Seminal vesical weight and prostate weight after 48 weeks of exposure with different treatment groups and control is presented in table 15. Significant reduction was observed in BPA 25 ug/L ($P < 0.05$) and BPA 50 ug/L ($P < 0.01$) when compared to the control. Absolute seminal vesical was reduced significantly ($P < 0.05$ and $P < 0.01$) in BPS 25, 50 ug/L treated groups. Similarly, BPF treatment caused significant reduction ($P < 0.05$ and $P < 0.01$) at does levels of 25 and 50 ug/L. On the other hand, BPS 25 and 50 ug/L significantly reduced ($P < 0.05$ and $P < 0.01$) absolute seminal vesicle weight; however, other doses of BPA, BPB, BPF and BPS did not reduce absolute seminal vesicle weight as compared to the control (Table 15).

Relative seminal vesicles weight of different treatment groups of BPA and its analogues BPB, BPF and BPS is presented in table 15. Significant reduction was observed in BPA 50 ug/L ($P < 0.01$) when compared to the control. Relative seminal vesicles weight was reduced significantly ($P < 0.01$) in BPB 50 ug/L treated group as compared to control group. Similarly, BPF treatment caused significant reduction ($P < 0.01$) at 50 ug/L dose level when comparison was done with the control. However, BPF 5 and 25 ug/L did not affect relative seminal weight significantly. BPS 50 ug/L relative seminal vesical weight significantly reduced ($P < 0.01$), however, other doses did not reduce relative seminal vesical weight as compared to the control (Table 15).

Absolute and relative prostate weight after 48 weeks of exposure with different concentration of BPA and its analogues BPB, BPF and BPS is presented in table 15. There was no significant difference observed in all the BPA and its analogues BPB, BPF and BPS treated groups as compared to the control. Prostate weight was observed to have reduced in some of the groups exposed to BPA and its analogues BPB, BPF and BPS but that reduction was not significant to the control (Table 15).

Table 14: Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 µg/L) on body and organs weight of rats

Treatments	Parameters				
	Final body weight (g)	**Paired testis (g)**	**GSI**	**Absolute Paired Epididymis (g)**	**Relative epididymis weight (mg/g)**
Control	541.11	3.68 ± 0.08	0.69 ± 0.03	1.44 ± 0.03	2.65 ± 0.03
BPA 5 ug/L	537.82	3.54 ± 0.05	0.65 ± 0.02	1.42 ± 0.02	2.62 ± 0.02
BPA 25 ug/L	538.43	3.53 ± 0.05	0.66 ± 0.03	1.40 ± 0.03	2.61 ± 0.03
BPA 50 ug/L	549.41*	3.50 ± 0.03	0.64 ± 0.01*	1.39 ± 0.01	2.55 ± 0.02**
BPB 5 ug/L	535.12	3.53 ± 0.04	0.67 ± 0.04	142 ± 0.04	2.61 ± 0.03
BPB 25 ug/L	537.60	3.55 ± 0.05	0.66 ± 0.03	141 ± 0.03	2.60 ± 0.02
BPB 50 ug/L	548.60*	3.49 ± 0.03	0.65 ± 0.02*	140 ± 0.02	2.54 ± 0.01**
BPF 5 ug/L	537.80	3.54 ± 0.04	0.68 ± 0.04	142 ± 0.03	2.62 ± 0.04
BPF 25 ug/L	538.41	3.53 ± 0.05	0.66 ± 0.03	141 ± 0.04	2.61 ± 0.03
BPF 50 ug/L	548.22*	3.51 ± 0.03	0.64 ± 0.02*	142 ± 0.02	2.55 ± 0.02**
BPS 5 ug/L	540.20	3.55 ± 0.04	0.67 ± 0.04	143 ±0.05	2.63 ± 0.03
BPS 25ug/L	538.60	3.54 ± 0.05	0.68 ± 0.03	142 ± 0.04	2.60 ± 0.02
BPS 50 ug/L	548.81*	3.50 ± 0.03	0.65 ± 0.01*	141 ± 0.02	2.56 ± 0.02**

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

Table 15: Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 µg/L) on the organs weight of rats

Treaments	Parameters			
	Absolute seminal vesicle weight (g)	Relative seminal vesicle weight (mg/g)	Absolute prostate weight (g)	Relative prostate weight (mg/g)
Control	1.90 ± 0.04	3.55 ± 0.04	1.45 ± 0.03	2.71 ± 0.05
BPA 5 ug/L	1.88 ± 0.03	3.48 ± 0.03	1.42 ± 0.03	2.69 ± 0.04
BPA 25 ug/L	1.82 ± 0.02 *	3.40 ± 0.04	1.46 ± 0.03	2.66 ± 0.03
BPA 50 ug/L	1.78 ± 0.03 **	3.30 ± 0.03 **	1.47 ± 0.04	2.65 ± 0.05
BPB 5 ug/L	1.86 ± 0.02	3.47 ± 0.03	1.43 ± 0.03	2.68 ± 0.03
BPB 25 ug/L	1.83 ± 0.03 *	3.41 ± 0.04	1.45 ± 0.02	2.67 ± 0.04
BPB 50 ug/L	1.79 ± 0.04 **	3.31 ± 0.02 **	1.46 ± 0.04	2.65 ± 0.02
BPF 5 ug/L	1.86 ± 0.02	3.46 ± 0.04	1.42 ± 0.03	2.67 ± 0.04
BPF 25 ug/L	1.82 ± 0.02 *	3.40 ± 0.03	1.44 ± 0.02	2.66 ± 0.03
BPF 50 ug/L	1.86 ±0.03 **	3.31 ± 0.03 **	1.41 ± 0.04	2.64 ± 0.04
BPS 5 ug/L	1.87 ± 0.02	3.49 ± 0.02	1.44 ± 0.03	2.67 ± 0.03
BPS 25ug/L	1.83 ± 0.03 *	3.42 ± 0.04	1.46 ± 0.02	2.68 ± 0.04
BPS 50 ug/L	1.79 ± 0.03 **	3.32 ± 0.03 **	1.48 ± 0.04	2.64 ± 0.03

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

***: Indicate significance at $p < 0.001$ vs control

Antioxidant enzymes, LPO and ROS after chronic exposure to different concentrations of BPA and its analogues BPB, BPF and BPS

Antioxidant enzymes reduced to a significant level while ROS and LPO levels increased in rat testicular tissues after chronic exposure to different concentrations of BPA and its analogues BPB, BPF and BPS as presented in table 16. CAT activity was expressed as unite/mg tissue and in BPA 25 µg/L and BPA 50 µg/L significant ($P < 0.05$) reduction was observed in exposed groups as compared to control group. Similarly, significant reduction was also observed in BPB 25 µg/L ($P < 0.05$) and BPB 50 µg/L ($P< 0.01$) groups when compared to the control group. On the other hand, CAT activity was significantly reduced in BPF 50 µg/L ($P < 0.05$) as compared to control. In BPS exposed group only significant reduction was observed in BPS 50 µg/L ($P < 0.05$) when compared to the control group. While there was no significant difference observed in the other exposed groups of BPA, BPB, BPF and BPS as compared to control. SOD activity was expressed as (mU/ mg protein) and in BPA 50 µg/L significant ($P < 0.01$) reduction was observed as compared to control. Similarly, BPB 50 µg/L exposed group caused significant ($P < 0.05$) reduction as compared to the control. On the other hand, BPF 50 µg/L significantly reduced ($P < 0.01$) SOD concentration in the rat testicular tissues. BPS high dose group 50 µg/L also ($P < 0.01$) reduced SOD concentration. However, 5 µg/L and 25 µg/L exposed groups did not show significant reduction in the SOD activity after chronic exposure with BPA, BPB, BPF and BPS. POD activity expressed as (U/mg protein) in the testis after chronic exposure, showed significant reduction in BPA 25 µg/L and 50 µg/L ($P < 0.05$ and $P < 0.01$) as compared to the control. Significant reduction was observed in BPB 25 µg/L ($P < 0.05$) and BPB 50 µg/L ($P < 0.01$) when compared to the control. POD activity was reduced significantly ($P < 0.05$ and $P < 0.01$) in BPF 25 µg/L and BPF 50 µg/L treated groups. Similarly, BPS treatment caused significant reduction ($P < 0.05$ and $P < 0.01$) at dose levels of 25 and 50 µg/L when compared to the control. However, BPA, BPB, BPF and BPS 5 µg/L did not affect POD activity significantly. LPO activity in the different treatment groups and control after chronic exposure is presented in table 4. Significant increase ($P < 0.01$) in BPA 50 µg/L was observed as compared to the control. All the high doses of BPB, BPF and BPS (50 µg/L) caused significant increase ($P < 0.01$) in the LPO activity as compared to control. However, there was no significant difference observed in 5 µg/L and 25 µg/L groups of BPA, BPB, BPF and BPS as compared to the control.

Table 16: Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 µg/L) on oxidative stress in the testicular tissues of rats

Treatments	Parameters				
	CAT (U/mg)	**SOD (U/mg)**	**POD (U/mg)**	**LPO (U/mg)**	**ROS (U/mg)**
Control	7.47 ± 0.15	32.34 ± 0.29	6.04 ± 0.15	7.72 ± 0.24	98.70 ± 0.29
BPA 5 ug/L	6.71 ± 0.41	32.09 ± 0.68	5.74 ± 0.07	7.62 ± 0.27	99.15 ± 0.18
BPA 25 ug/L	6.43 ± 0.25*	31.38 ± 0.43	5.60 ± 0.09*	7.73 ± 0.02	104.5 ± 1.67
BPA 50 ug/L	6.38 ± 0.25*	30.66 ± 0.33**	5.40 ± 0.10**	8.43 ± 0.07**	122.7 ± 3.53***
BPB 5 ug/L	7.11 ± 0.35	32.16 ± 0.30	5.65 ± 0.04	7.49 ± 0.07	98.35 ± 0.42
BPB 25 ug/L	6.38 ± 0.30*	31.34 ± 0.31	5.50 ± 0.13*	7.57 ± 0.08	105.0 ± 2.73
BPB 50 ug/L	6.09 ±0.28**	30.81 ± 0.20*	5.42 ± 0.07**	8.60 ± 0.22**	122.6 ± 3.34***
BPF 5 ug/L	7.13 ± 0.13	32.32 ± 0.24	5.65 ± 0.05	7.38 ± 0.06	98.70 ± 0.42
BPF 25 ug/L	6.46 ± 0.27	31.14 ± 0.30	5.54 ± 0.11*	7.54 ± 0.09	105.4 ± 1.12
BPF 50 ug/L	6.17 ±0.24**	30.42 ± 0.11**	5.41 ± 0.13**	8.59 ± 0.14**	122.0 ± 4.06***
BPS 5 ug/L	7.08 ± 0.26	32.59 ± 0.17	5.62 ± 0.09	7.48 ± 0.10	98.84 ± 0.40
BPS 25ug/L	6.46 ± 0.20	31.63 ± 0.16	5.45 ± 0.09*	7.56 ± 0.08	105.4 ± 1.37
BPS 50 ug/L	6.36 ± 0.16*	30.57 ± 0.15**	5.44 ± 0.11**	8.60 ± 0.03**	121.5 ± 3.28***

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

***: Indicate significance at $p < 0.001$ vs control

Plasma testosterone, Luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol concentrations in the animals after chronic exposure of 48 weeks to different concentrations of BPA and its analogues BPB, BPF and BPS

Plasma testosterone (ng/ml), LH (ng/ml), FSH (mIU/ml) and estradiol concentrations (ph/ml) are presented in table 17. Significant reduction was observed in the testosterone concentrations of BPA 50 µg/L ($P < 0.01$) when compared to the control group. Testosterone concentrations reduced significantly ($P < 0.001$) in BPB 50 µg/L treated group as compared to the control. Similarly, BPF caused significant reduction ($P < 0.001$) at dose level 50 µg/L as compared to the control group. On the other hand, BPS 25 µg/L and 50 µg/L significantly reduced ($P < 0.05$ and $P < 0.001$ respectively) testosterone in the plasma, however other doses of BPA, BPB, BPF and BPS did not reduce plasma testosterone as compared to the control.

Plasma estradiol concentrations in the animals exposed to BPA 50 µg/L were significantly ($P < 0.05$) increased than control group. Estradiol concentrations increased significantly ($P < 0.01$) in BPB 50 µg/L treated group as compared to control. Similarly, BPF treatment caused significant increase ($P <0.001$) at dose level of 50 µg/L, however, BPF 5 µg/L and 25 µg/L did not affect estradiol concentration significantly. On the other hand, BPS 50 µg/L significantly increased ($P < 0.001$) estradiol concentrations; however, other groups did not cause any increase in estradiol concentration as compared to the control.

Plasma LH concentrations in the treatment groups were found reduced as compared to the control group presented in table no 17. Significant reduction was observed in BPA 50 µg/L ($P < 0.05$) when compared to the control group. LH concentrations was reduced significantly ($P < 0.05$) in BPB 50 µg/L treated group in comparison to the control group. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose level of 50 µg/L. BPS 50 µg/L significantly reduced ($P < 0.05$) plasma LH concentrations, however, other doses did not reduce plasma LH concentrations as compared to the control.

Plasma FSH concentrations in the treatment groups were found reduced as compared to the control group presented in table 17. Significant reduction in plasma FSH levels ($P < 0.05$) was noted in the highest concentrations (50 µg/L) exposed group of BPA when compared to the control. FSH concentrations was reduced significantly ($P < 0.05$) in BPB 50 µg/L when compared to the control. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose

level of 50 μg/L as compared to the control group. On the other hand, PBS 50 μg/L significantly reduced ($P < 0.05$) FSH concentration in plasma. However, other treatment groups of BPA, BPB, BPF and BPS plasma FSH levels were reduced but were not statistically significant.

Sperm parameters, DSP and number of sperm in different parts of epididymis after chronic exposure to different concentrations of BPA and its analogues BPB, BPF and BPS

Exposure to different concentration of BPA and its analogues BPB, BPF and BPS for 48 weeks caused no significant reduction in the percentage of motile sperm presented in table 18. However, highest concentrations of BPA (50 μg/L) for 48 weeks caused significant reduction ($P < 0.05$) on the percentage motile sperm but did not show any effect on the percentage viable sperm. Significant reduction was observed in BPB 50 μg/L ($P < 0.01$) when compared to control. Motile sperm percentage was reduced significantly ($P < 0.05$ and $P < 0.01$) in BPF 25 and 50 μg/L. On the other hand, PBS 25 and 50 μg/L significantly reduced ($P < 0.05$ and $P < 0.01$) percentage of motile sperms after exposure for 48 weeks of chronic exposure. However, in the different concentrations of BPA, BPB, BPF and BPS where no significant difference observed when compared to control (Table 18).

DSP in the different groups is presented in table 18. Significant reduction was observed in BPA 50 μg/L ($P < 0.01$) when compared to control. DSP was reduced significantly ($P < 0.01$) in BPB 50 μg/L treated group. Similarly, BPF treatment caused significant reduction ($P < 0.01$) at dose level of 50 μg/L. BPS 50 μg/L also caused significant reduction ($P < 0.01$) in the treated groups. On the other hand, other doses of bisphenols did not cause any significant effect on the daily sperm production.

Table 17: Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 µg/L) on plasma testosterone, estradiol, LH and FSH concentrations in rats

Treatments	Plasma hormone concentrations			
	Testosterone (ng/ml)	Estradiol (pg/ml)	LH (ng/ml)	FSH (mIU/ml)
Control	12.02 ± 0.98	2.81 ± 0.33	1.79 ± 0.07	0.79 ± 0.07
BPA 5 ug/L	11.68 ± 0.43	3.64 ± 0.24	1.68 ± 0.08	0.75 ± 0.02
BPA 25 ug/L	10.61 ± 020	3.72 ± 0.40	1.55 ± 0.08	0.67 ± 0.04
BPA 50 ug/L	09.76 ± 0.36**	4.20 ± 0.34*	1.52 ± 0.03*	0.59 ± 0.05*
BPB 5 ug/L	11.05 ± 0.23	3.47 ± 0.19	1.62 ± 0.04	0.76 ± 0.07
BPB 25 ug/L	10.90 ± 0.21	3.93 ± 0.22	1.55 ± 0.03	0.63 ± 0.06
BPB 50 ug/L	09.36 ± 0.41***	4.55 ± 0.33**	1.48 ± 0.02*	0.58 ± 0.05*
BPF 5 ug/L	11.49 ± 0.37	3.53 ± 0.19	1.59 ± 0.08	0.73 ± 0.04
BPF 25 ug/L	10.43 ± 0.33	3.86 ± 0.26	1.54 ± 0.05	0.64 ± 0.01
BPF 50 ug/L	09.40 ± 0.05***	4.48 ± 0.29**	1.49 ± 0.07*	0.59 ± 0.02*
BPS 5 ug/L	11.39 ± 0.11	3.43 ± 0.31	1.63 ± 0.06	0.74 ± 0.03
BPS 25ug/L	10.31 ± 0.63*	3.82 ± 0.16	1.56 ± 0.06	0.60 ± 0.02
BPS 50 ug/L	09.45 ± 0.33***	4.39 ± 0.29**	1.49 ± 0.02*	0.58 ± 0.03*

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

***: Indicate significance at $p < 0.001$ vs control

Table 18: Effect of chronic exposure of different concentrations of BPA and its alternatives BPB, BPF and BPS (5, 25 and 50 µg/L) on sperm parameters and sperm number in epididymis of rats

Treatments	Parameters				
	Viable sperms (%)	Motile sperms (%)	DSP (x 106)	Caput epididymis sperm number (× 106/g organ)	Cauda epididymis sperm number (× 106/g organ)
Control	93.92 ± 0.48	79.56 ± 0.54	53.34 ± 0.6	303.16 ± 1.38	598.15 ± 2.46
BPA 5 ug/L	93.87 ± 0.65	77.72 ± 1.74	52.22 ± 0.3	296.62 ± 3.88	590.57 ± 0.22
BPA 25 ug/L	93.52 ± 0.92	77.01 ± 1.69	50.56 ± 1.4	291.78 ± 2.03*	589.28 ± 4.88
BPA 50 ug/L	92.01 ± 0.89	77.27 ± 0.89*	48.44 ± 0.3**	291.88 ± 4.11**	583.38 ±1.64*
BPB 5 ug/L	93.95 ± 0.84	78.08 ± 0.68	52.34 ± 0.7	295.04 ± 2.10	592.18 ± 2.10
BPB 25 ug/L	93.13 ± 0.74	75.97 ± 0.51	51.04 ± 1.5	293.92 ± 2.04*	590.38 ± 5.06
BPB 50 ug/L	92.33 ± 0.86	74.17 ± 0.42**	48.32 ± 0.5**	290.16 ± 1.12**	580.98 ± 0.94*
BPF 5 ug/L	93.49 ± 0.97	78.33 ± 0.34	52.14 ± 0.6	295.14 ± 2.05	592.46 ± 2.02
BPF 25 ug/L	93.13 ± 1.09	75.33 ± 0.38*	50.68 ± 1.1	293.28 ± 0.75*	589.36 ± 2.66
BPF 50 ug/L	92.19 ± 0.91	74.70 ± 0.30**	48.58 ± 0.7**	288.86 ± 0.96**	583.14 ± 1.66*
BPS 5 ug/L	93.57 ± 1.07	78.12 ± 0.51	52.24 ± 0.5	295.52 ± 1.55	590.74 ± 5.07
BPS 25ug/L	93.32 ± 1.01	75.27 ± 1.10*	50.32 ± 0.8	293.48 ± 1.77*	589.94 ± 4.88
BPS 50 ug/L	92.99 ± 0.97	74.28 ± 0.74**	48.22 ± 0.5**	291.12 ± 1.70**	584.64 ± 1.68*

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

Sperm number in caput epididymis was significantly reduced in the BPA 25 μg/L ($P < 0.05$) and BPA 50 μg/L ($P < 0.01$) exposed groups as compared to the control group. Significant reduction was observed in BPB 25 μg/L ($P < 0.05$) and BPB 50 μg/L ($P < 0.01$) when compared to the control. Similarly, BPF treatment caused reduction ($P < 0.05$ and $P < 0.01$) at dose levels of 25 and 50 μg/L. In BPS 25 and 50 μg/L significant reduction ($P < 0.05$ and $P < 0.01$) was observed in the caput epididymis sperm number when compared to the control group. However, some of the BPA, BPB, BPF and BPS did not reduce sperm number in the caput epididymis as compared to the control.

Sperm number in the cauda epididymis in different treatment groups and control is presented in table 18. Significant reduction was observed in BPA 50 μg/L ($P < 0.05$) when compared to the control. Cauda epididymis sperm number was reduced significantly ($P < 0.05$) in BPB 50 μg/L treated group in comparison to the control group. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose level of 50 μg/L as compared to the control group. BPS 50 μg/L also significantly reduced ($P <0.05$) cauda epididymis sperm number as compared to control. On the other hand, there was no significant difference observed in BPA, BPB, BPF and BPS 5 and 25 μg/L treated groups when compared to the control.

Histopathological and planimetry changes in the testicular tissue of adult male rats exposed to different concentrations of BPA and its analogues BPB, BPF and BPS after 48 weeks of exposure in male rats

Histological study of the microscopic slides of the testicular tissues revealed normal morphology of the structures in the control and 5 μg/L exposed groups. The seminiferous tubules were compactly arranged with sperm filled lumen and the interstitial space was relatively thin in the exposed groups in comparison to the control group. In the groups exposed to 25 μg/L and 50 μg/L of BPA and its analogues BPB, BPF and BPS the tubules were relatively small with larger interstitial spaces and less filled lumen. Cellular arrest at spermatogoneal stage and round spermatids were more evident in the highest concentration (50 μg/L) exposed group. In 25 μg/L exposed group, cellular arrest was observed but was less than 50 μg/L exposed group (Fig 11).

Planimetry results showed significant ($P < 0.05$) reduction in the height of seminiferous epithelium in the group exposed to 50 μg/L of BPA for weeks. Significant reduction was

observed in BPB 50 μg/L ($P < 0.01$) when compared to the control. Epithelial height was reduced significantly ($P < 0.01$) in BPF 50 μg/L treated group. Similarly, BPS treatment caused significant reduction ($P < 0.05$) at dose level of 50 μg/L. However, BPA, BPB, BPF and BPS 5 and 25 μg/L groups did not affect epithelial height significantly. On the other hand, there was no significant difference observed in area of seminiferous tubules, area of interstitium and in diameter of seminiferous tubules of all treated groups of BPA, BPB, BPF and BPS as compared to the control (Table 19).

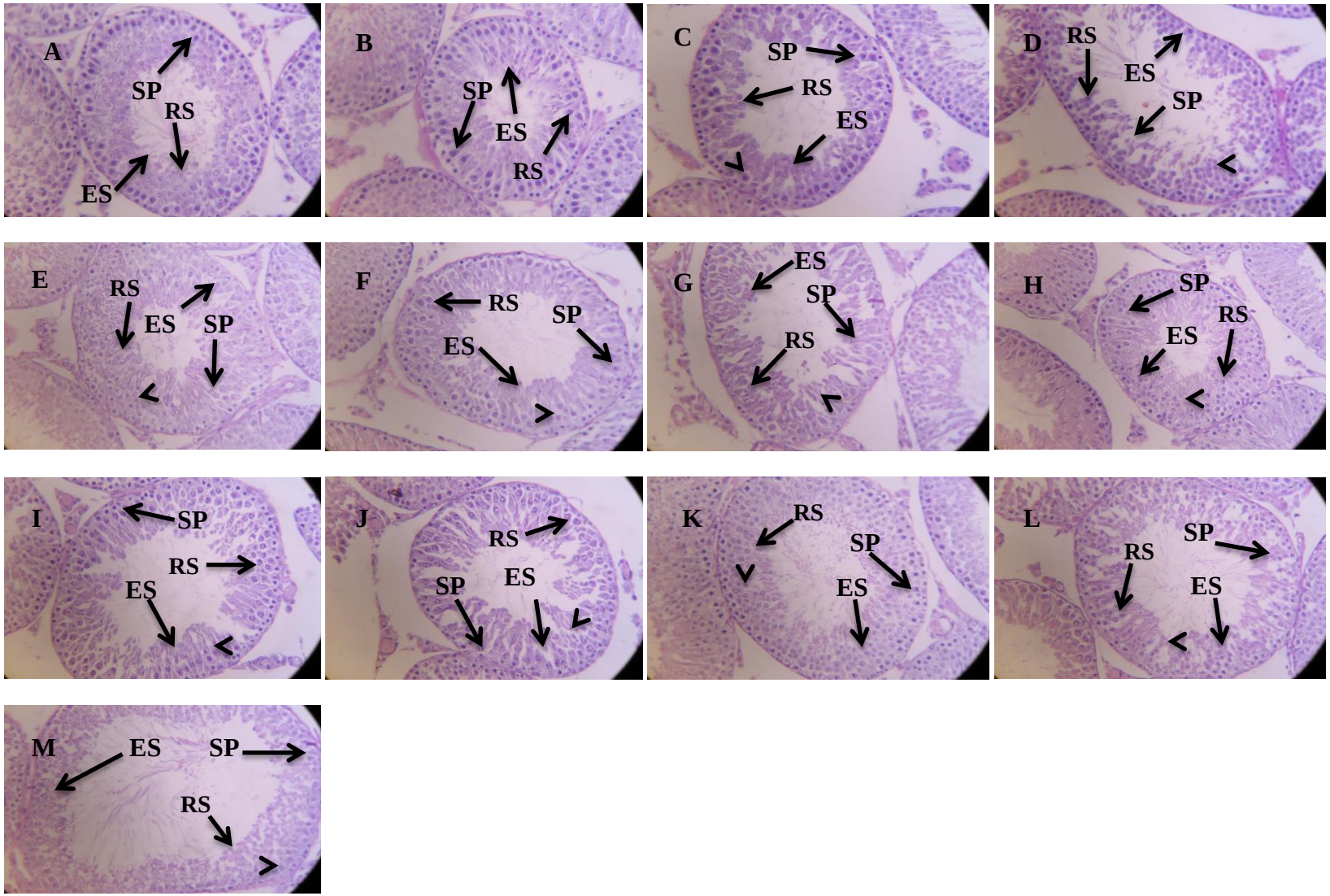

Fig 11: Photomicrograph from testicular tissue showing (A) control; having thick epithelium with normal spermatogonia (SP), Round spermatids (RS), Elongated spermatids (ES) and filled lumen with sperm (B, C and D); BPA (5, 25 and 50 µg/L) treated presenting seminiferous tubules with epithelium (line without arrow head) and spermatids (white arrow); (E, F and G)

BPB (5, 25 and 50 µg/L) treated presenting seminiferous tubules with epithelium (line without arrow head) and elongating spermatids (white arrow); (H, I and J) BPF (5, 25 and 50 µg/L) treated presenting seminiferous tubules with epithelium (line without arrow head) and elongating spermatids (white arrow); (K, L and M) BPS (5, 25 and 50 µg/L) treated presenting seminiferous tubules with epithelium (line without arrow head) and spermatids (white arrow). H&E (x40)

Table 19: Effect of chronic exposure of different concentrations of BPA and its alternatives BPB, BPF and BPS (5, 25 and 50 µg/L) on planimetry of testis in rats

Treatments	Parameters			
	Area of seminiferous tubules (%)	**Area of Interstitium (%)**	**Seminiferous tubule diameter (µm)**	**Epithelial height (µm)**
Control	85.02 ± 1.95	16.42 ± 0.72	207.90 ± 1.77	71.22 ± 1.90
BPA 5 ug/L	82.64 ± 0.23	17.80 ± 0.95	201.08 ± 3.13	67.88 ± 1.02
BPA 25 ug/L	82.06 ± 0.67	16.22 ± 1.32	205.08 ± 1.55	65.74 ± 1.28
BPA 50 ug/L	82.17 ± 1.72	16.66 ± 1.38	203.97 ± 1.48	61.58 ± 2.17*
BPB 5 ug/L	82.73 ± 1.05	17.68 ± 0.38	205.87 ± 1.60	69.18 ± 1.29
BPB 25 ug/L	81.64 ± 0.56	15.90 ± 1.49	207.46 ± 1.47	68.13 ± 1.31
BPB 50 ug/L	83.71 ± 1.38	15.69 ± 1.37	203.24 ± 1.25	60.02 ± 2.72**
BPF 5 ug/L	84.58 ± 1.54	16.26 ± 1.63	204.81 ± 1.59	68.06 ± 2.10
BPF 25 ug/L	82.44 ± 0.71	15.65 ± 1.29	203.53 ± 1.72	66.35 ± 1.75
BPF 50 ug/L	84.46 ± 1.26	17.02 ± 1.51	205.46 ± 1.22	60.83 ± 2.15**
BPS 5 ug/L	83.51 ± 0.82	18.20 ± 0.52	205.24 ± 1.24	66.26 ± 2.65
BPS 25 ug/L	82.30 ± 0.69	17.86 ± 0.66	204.86 ± 1.58	64.44 ± 1.87
BPS 50 ug/L	83.28 ± 0.71	19.04 ± 0.78	204.35 ± 1.63	61.96 ± 2.72*

Values are presented as mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

***: Indicate significance at $p < 0.001$ vs control

Number of different cells types in seminiferous tubules in the testis of adult male rats exposed to different concentrations of BPA and its analogues BPB, BPF and BPS for 48 weeks

Total number of different cells population present in the seminiferous tubules of rats testis is presented in table 20. Significant reduction in the number of spermatogonia was observed in the group exposed to BPA 50 μg/L ($P < 0.05$) than control. Significant reduction was also observed in BPB 50 μg/L ($P < 0.05$) when compared to the control. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose level of 50 μg/L as compared to the control group. On the other hand, BPS 50 μg/L significantly reduced ($P < 0.05$) number of spermatogonia as compared to control. However, BPA, BPB, BPF and BPS 5 and 25 μg/L did not significantly reduce number of spermatogonia as compared to control.

In the number of spermatocytes significant reduction was observed in BPA 50 μg/L ($P < 0.05$) when compared to the control. Spermatocytes number was reduced significantly ($P < 0.05$) in BPB 50 μg/L treated group as compared to the control group. Similarly, BPF 50 μg/L treatment caused significant reduction ($P < 0.05$) at dose level of 50 μg/L. BPS 50 μg/L treated group significantly reduced ($P < 0.05$) the number of spermatocytes when compared to the control. On the other hand, the other doses of BPA, BPB, BPF and BPS did not reduce number of spermatocytes as compared to the control.

Number of spermatids in different treatment groups and control is presented in table 20. Significant reduction was observed in BPA 50 μg/L ($P < 0.01$) when compared to the control. Number of spermatids reduced significantly ($P < 0.01$) in BPB 50 μg/L treated group. Similarly, BPF treatment caused significant reduction ($P < 0.01$) at dose level of 50 μg/L as compared to the control. BPS 50 μg/L group was also observed with significantly reduced ($P < 0.01$) number of spermatids as compared to the control. However, there was no significant difference observed in BPA, BPB, BPF and BPS 5 and 25 μg/L groups when compared to the control.

Table 20. Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 µg/L) on number of different cell types in the testis of rats

	Parameters		
Groups	**Spermatogonia (n)**	**Spermatocytes (n)**	**Spermatids (n)**
Control	65.66 ± 0.62	77.10 ± 1.06	257.26 ± 1.79
BPA 5 ug/L	63.14 ± 0.75	75.40 ± 1.29	250.54 ± 2.67
BPA 25 ug/L	63.56 ± 0.83	73.32 ± 1.97	248.10 ± 2.71
BPA 50 ug/L	60.62 ± 0.72*	72.18 ± 1.20*	245.58 ± 2.42**
BPB 5 ug/L	63.98 ± 1.36	74.32 ± 0.94	250.32 ± 1.80
BPB 25 ug/L	63.68 ± 1.03	73.54 ± 1.41	248.36 ± 2.20
BPB 50 ug/L	61.26 ± 1.13*	71.82 ± 1.29*	245.40 ± 2.50**
BPF 5 ug/L	63.72 ± 1.13	73.64 ± 1.35	250.10 ± 2.87
BPF 25 ug/L	63.20 ± 1.16	72.64 ± 1.24	248.22 ± 2.34
BPF 50 ug/L	61.34 ± 0.84*	71.50 ± 1.26*	245.16 ± 1.97**
BPS 5 ug/L	63.40 ± 1.05	74.74 ± 1.30	250.04 ± 2.77
BPS 25ug/L	63.64 ± 1.15	73.84 ± 1.23	248.32 ± 2.52
BPS 50 ug/L	61.58 ± 0.87*	72.12 ± 1.24*	244.02 ± 2.01**

Values are presented as mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

Planimetry and morphological changes in the caput region of epididymis of rats exposed to different concentrations of BPA and its analogues BPB, BPF and BPS for 48 weeks

Epididymis caput region planimetry results did not show any significant reduction in the tubular diameter in the groups exposed to different concentrations of BPA and its analogues BPB, BPF and BPS after 48 weeks of chronic exposure. There was also no significant difference observed in the other parameters as lumen diameter, epithelial height, area covered with epithelium and lumen of different treatment groups when compared to the control (Table 21, Figure 12).

There was very slight difference observed in the morphology of caput region of epididymis among the different treatment groups of BPA and its analogues BPB, BPF and BPS and control. In the different treatment groups (50 μg/L) of BPA and its analogues BPB, BPF and BPS slightly reduced number of sperm in the lumen was observed when compared to the control. There was no significant difference observed in the other exposed groups in comparison to the control (Table 21).

Planimetry and morphological changes in the cauda region of epididymis of rats exposed to different concentrations of BPA and its analogues BPB, BPF and BPS for 48 weeks

Planimetry of the cauda region of the epididymis showed no significant alterations in the tubular diameter in the groups exposed to different concentrations of BPA and its analogues BPB, BPF and BPS than control after 48 weeks of exposure. Similarly, other parameters like lumen diameter, epithelial height, area covered by epithelium and area covered by lumen did not show any significant alterations compared to the control (Table 22, Fig 13). Morphological difference observed in the cauda region of epididymis showed only a slightly reduced number of sperm in the lumen in 50 μg/L exposed with different concentrations of BPA, BPB, BPF and BPS for 48 weeks of chronic exposure. No significant alterations were obvious in other treated groups in comparison with control as presented in fig 13.

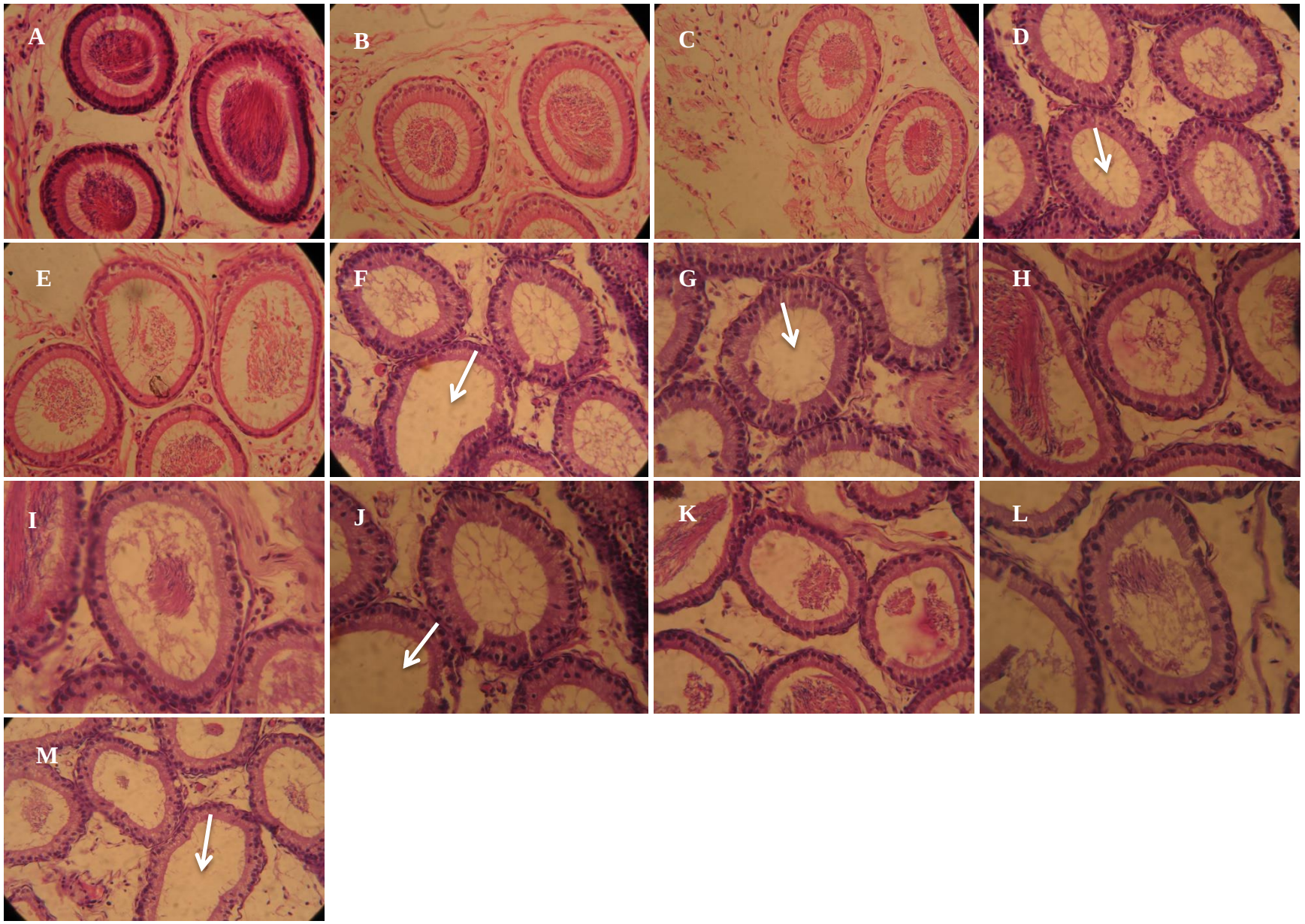
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Fig 12: Photomicrograph of caput epididymis tissue showing (A) control; with compact arrangement of caput tubules with sperm filled lumen (B) BPA (5 µg/L) exposed group, presenting normal caput tubules like in the control (C), BPA (25 µg/L) exposed group showing seminiferous tubules with less number of sperm in the lumen (Arrow) and (D) BPA (50 µg/L) exposed group presenting caput tubules with empty lumen (Arrow). Similarly, (E) BPB (5 µg/L) exposed group, presenting normal caput tubules, (F) BPB (25 µg/L) exposed group showing less number of sperms in the lumen, (G) BPB (50 µg/L) exposed group showing less number of sperms and empty lumen (Arrow). (H) BPF (5 µg/L) exposed group, presenting normal caput tubules, (I) (25 µg/L) exposed group showing seminiferous tubules with less number of sperm in the lumen (Arrow) and (J) BPF (50 µg/L) exposed group showing less number of sperms and empty lumen (Arrow). K and L BPS (5 and 25 µg/L) exposed groups showing caput tubules with less number of sperms in the lumen and (M) BPS (50 µg/L) exposed group presenting less number of sperms and empty lumen. H&E (x40).

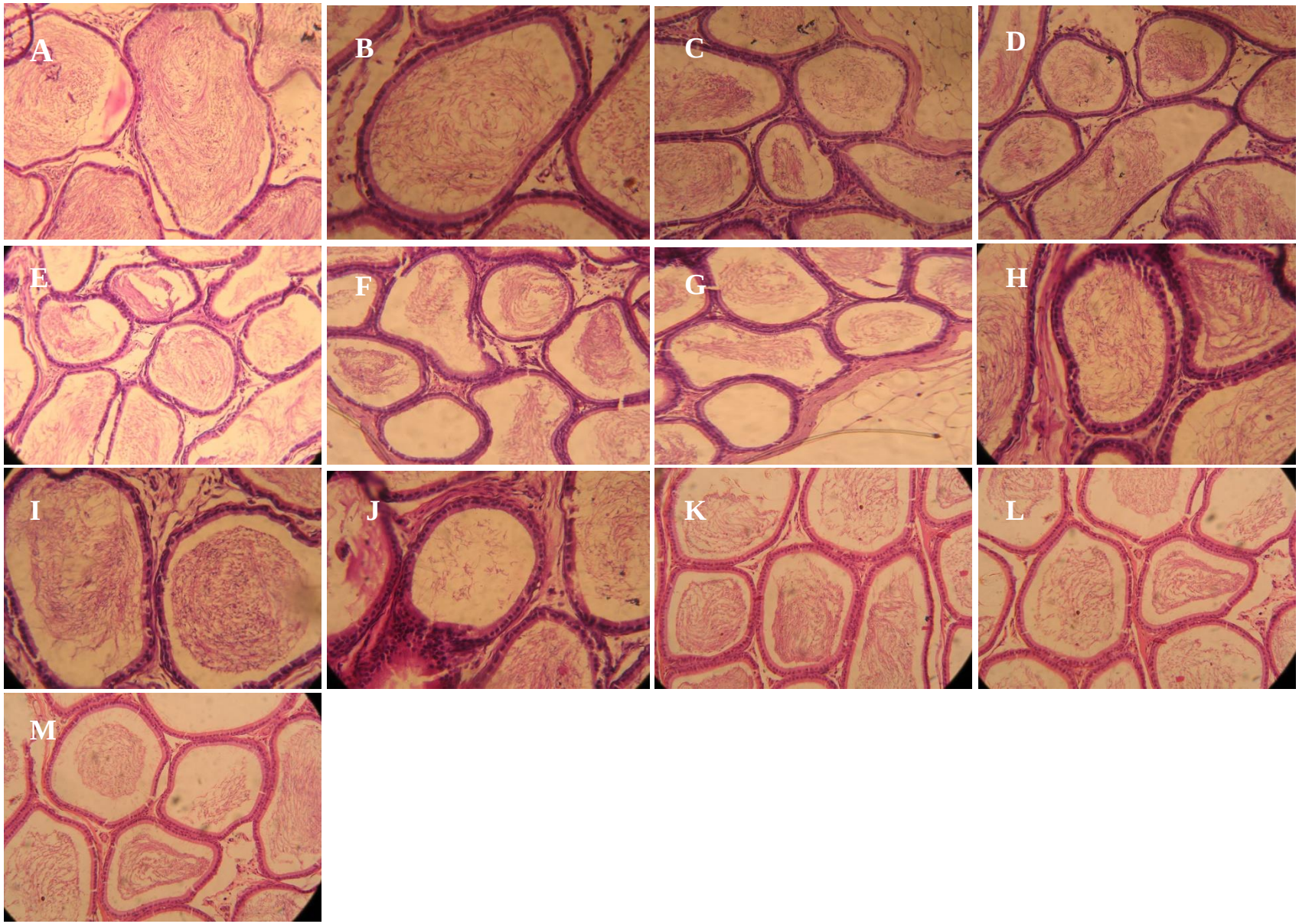
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Fig 13: Photomicrograph of cauda epididymis tissue showing (A) control; with compact arrangement of cauda tubules with sperm filled lumen (B) BPA (5 µg/L) exposed group, presenting normal caput tubules like in the control (C) BPA (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (D) BPA (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. Similarly, (E) BPB (5 µg/L) exposed group, presenting normal caput tubules like in the control (F) BPB (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (G) BPB (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. Likewise, (H)BPF (5 µg/L) exposed group, presenting normal caput tubules like in the control (I) BPF (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (J) BPF (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. In the same way, (K) BPS (5 µg/L) exposed group, presenting normal caput tubules like in the control; (L) BPS (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (M) BPS (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. H&E (x40).

Table 21. Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 µg/L) on planimetry of caput epididymis in rats

Treaments	Parameters				
	Tubular diameter (µm)	Lumen daimeter (µm)	Epithelial height (µm)	Epithelium (%)	Lumen (%)
Control	366.40 ± 1.34	292.01 ± 2.76	34.05 ± 1.03	33.25 ± 2.37	70.75 ± 4.70
BPA 5 ug/L	358.80 ± 1.75	290.60 ± 2.61	33.40 ± 2.43	32.05 ± 1.50	69.75 ± 1.94
BPA 25 ug/L	356.20 ± 3.21	288.02 ± 1.90	30.04 ± 2.79	31.51 ± 0.49	68.55 ± 2.00
BPA 50 ug/L	357.20 ± 3.05	287.20 ± 2.22	29.40 ± 1.01	29.25 ± 2.49	64.25 ± 2.86
BPB 5 ug/L	359.04 ± 2.19	290.60 ± 1.70	33.04 ± 0.44	32.98 ± 1.06	69.55 ± 4.33
BPB 25 ug/L	358.40 ± 4.99	288.20 ± 1.48	31.40 ± 2.26	31.65 ± 0.48	68.75 ± 4.67
BPB 50 ug/L	357.80 ± 3.03	287.80 ± 0.95	30.75 ± 2.49	29.16 ± 1.13	65.75 ± 2.78
BPF 5 ug/L	358.40 ± 0.74	290.80 ± 1.96	33.05 ± 2.42	32.65 ± 2.17	67.95 ± 1.70
BPF 25 ug/L	359.20 ± 1.57	288.60 ± 0.24	31.40 ± 1.75	31.05 ± 1.83	65.25 ± 0.98
BPF 50 ug/L	356.80 ± 3.27	287.20 ± 2.47	30.05 ± 0.88	29.20 ± 1.13	64.35 ± 3.58
BPS 5 ug/L	357.60 ± 1.27	290.60 ± 2.98	33.60 ± 1.81	33.40 ± 1.58	68.95 ± 1.42
BPS 25ug/L	355.80 ± 2.19	289.80 ± 4.63	31.20 ± 3.07	30.50 ± 2.39	66.05 ± 0.72
BPS 50 ug/L	354.40 ± 3.13	287.80 ± 3.02	30.40 ± 2.47	28.50 ± 1.49	65.75 ± 1.60

Table 22: Effect of chronic exposure of different concentrations of BPA and its analogues BPS, BPF and BPS (5, 25 and 50 µg/L) on planimetry of cauda epididymis in rats

Treatments	Parameters				
	Tubular diameter (µm)	Lumen diameter (µm)	Epithelial height (µm)	Epithelium (%)	Lumen (%)
Control	443.61 ± 1.67	415.60 ± 2.13	28.65 ± 1.05	33.25 ± 2.94	67.75 ± 1.97
BPA 5 ug/L	440.81 ± 0.72	412.60 ± 1.38	27.53 ± 1.46	31.51 ± 2.08	68.11 ± 0.88
BPA 25 ug/L	440.61 ± 3.91	411.11 ± 2.98	26.72 ± 0.86	28.91 ± 0.70	67.31 ± 1.68
BPA 50 ug/L	439.81 ± 2.32	410.10 ± 2.98	26.22 ± 1.75	27.75 ± 6.66	70.05 ± 1.69
BPB 5 ug/L	439.81 ± 0.95	413.40 ±1.73	27.62 ± 1.45	29.51 ± 0.72	68.31 ± 2.27
BPB 25 ug/L	440.81 ± 2.95	415.60 ± 2.35	26.28 ± 1.68	27.25 ± 1.13	68.75 ± 1.87
BPB 50 ug/L	439.81 ± 3.11	414.60 ± 1.96	25.62 ± 2.10	26.75 ± 2.00	70.45 ± 1.27
BPF 5 ug/L	440.01 ± 0.54	414.40 ± 0.91	27.82 ± 2.45	31.51 ± 2.29	68.51 ± 2.00
BPF 25 ug/L	439.81 ± 1.22	413.20 ± 1.80	26.82 ± 2.39	29.75 ± 6.36	70.25 ± 1.67
BPF 50 ug/L	439.81 ± 1.13	413.12 ± 1.90	26.21 ± 1.00	27.51 ± 6.36	70.51 ± 3.55
BPS 5 ug/L	440.61 ± 2.13	414.13 ± 4.32	27.21 ± 2.19	29.51 ± 2.39	68.51 ± 2.54
BPS 25ug/L	440.21 ± 1.05	413.80 ± 1.63	26.80 ± 3.10	27.75 ± 1.26	68.85 ± 1.17
BPS 50 ug/L	441.81 ± 1.75	413.40 ± 1.73	25.60 ± 3.24	27.51 ± 1.17	70.51 ± 3.55

DISCUSSION

A large number of studies recently have reported the adverse toxic effects of BPA involvement in many chronic diseases. Therefore, the concerns of many environmental agencies and government security groups have led to the development of many substitutes for BPA such BPB, BPF and BPS. These all analogues leaching from plastic containers have been shown to a lesser extent; though it has been detected in a greater amount in the food samples across the globe (Viñas *et al.*, 2010, Liao and Kannan, 2013, Liao and Kannan, 2014b, Liao and Kannan, 2014a, Yamazaki *et al.*, 2015). Although there is very little data on the effects of low dose of BPA and its analogues BPB, BPF and BPS which are widely used to replace BPA in the modern world of plastic industry. Widespread use of BPA and its analogues has caused concerns over the adverse effects provoked by these substances on human health (Song *et al.*, 2014a). *In vitro* and *in vivo* studies and epidemiological surveys have shown that BPA and its analogues exhibits neurotoxic potentials, hepatotoxic effects, cancer development risk and endocrine toxicity (Cabaton *et al.*, 2009, Soto *et al.*, 2013, Catanese and Vandenberg, 2016, Grignard *et al.*, 2012, Rochester and Bolden, 2015). There has been less attention given to BPA analogues and its toxicological effects on reproductive system.

The aim of this study is to assess the toxic effects of BPA and its analogues BPB, BPF and BPS on reproductive system of male rat for duration of 48 weeks in comparison with the effects of BPA. In this study we have shown that BPB, BPF and BPS have many properties in common to BPA where we observed reduction in GSI, relative weights of reproductive organs, testosterone, LH and FSH concentrations and alterations in the reproductive tissues histology in groups exposed to higher concentrations of BPA and its analogues BPB, BPF and BPS in comparison to the control. Oxidative stress in the testicular tissues was observed and the DSP was reduced in the higher concentrations exposed groups than control. Our results were not very different from some of these studies done in past with BPA and its analogues (Rubin, 2011, Völkel *et al.*, 2002, Meeker *et al.*, 2009a, Shi *et al.*, 2015).

In the present study process of steroidogenesis got disturbed after exposure to BPA and its analogues. LH and FSH concentrations were inhibited and the concentrations of testosterone had decreased significantly in the exposed groups. However, the concentrations of estradiol in exposed groups had increased which suggest that either the gonadotropin secretions were inhibited at the level of pituitary or the secretions of GnRH from hypothalamus were affected

which resulted in reduced levels of testosterone. This can also be because of the disturbance resulted by prolonged oxidative stress in the testicular tissues as reported earlier (Moghaddam *et al.*, 2015, Hassan *et al.*, 2012, Feng *et al.*, 2016, Naderi *et al.*, 2014, Yang *et al.*, 2017a). Previously, it was reported that BPA and BPS exposure lead to oxidative stress in the peripheral blood mononuclear cells and testis and also lead to lipids and protein degradation (Michałowicz *et al.*, 2015, Ullah *et al.*, 2017, Ullah *et al.*, 2016, Mokra *et al.*, 2015). The results of our study about inhibition of testosterone and anti-androgenic effects of these chemicals are in line with studies of (Rochester and Bolden, 2015, Molina-Molina *et al.*, 2013). Testosterone reduced concentrations might be a result of suppression of GnRH transcripts in the hypothalamus which also suggest that suppressed GnRH leads in reduced gonadotropin secretion (Ji *et al.*, 2013, Roelofs *et al.*, 2015). However, increased estrogen levels seem to be due to estrogenic mode of action of BPA and its analogues BPB, BPF and BPS (Sui *et al.*, 2012, Liao and Kannan, 2013, Yamazaki *et al.*, 2015).

Besides, the reduction in the LH and FSH levels we observed reduced testosterone concentrations, reduced DSP and number of sperms in epididymis exposed to different concentrations of BPA and its analogues BPB, BPF and BPS. Similarly, the reproductive organs weights were also reduced in groups exposed to different concentrations of BPA and its analogues. Our results are in accordance with the previous studies where BPA and its analogues have been observed to cause an increase in the adipogenesis and preadipocytes (Somm *et al.*, 2009, Héliès-Toussaint *et al.*, 2014, Ahmed and Atlas, 2016). BPA and its analogues have also been observed to be associated with obesity and high fat in the different organs (Boucher *et al.*, 2016b, Del Moral *et al.*, 2016, Vom Saal *et al.*, 2012, Somm *et al.*, 2009).

Poor developments of reproductive organs lead to reduction in the daily sperm production, reduction in the GSI of rats and alteration in the morphology of seminiferous tubules. The reduction in these parameters in our study were accompanied by arrest in spermatogoneal cells and round spermatids, which seem to have resulted because of reduced DSP, reduced number of sperm in the epididymis and epithelial height. Our results are in relation with multiple studies with BPA and some of its analogues where LH and FSH reduced levels supported the histological alterations in the testis and reduction in sperm production (Somm *et al.*, 2009, Brown *et al.*, 2008, Chen *et al.*, 2013, Eladak *et al.*, 2015). Previous literature has also shown that estrogenic compounds do have effects on the reducing weight of the reproductive organs in

the adulthood. The main reason for the reduction in weight and spermatogenesis is the presence of androgen and estrogen receptors in these organs that paly critical role in the spermatogenesis. On the other hand, gonadotropin receptor is also considered very important in the synthesis of androgens and spermatogenesis. It has been reported in several studies that any sort of alteration in these receptors lead into alteration in the testis physiology and spermatogenesis (Blake and Ashiru, 1997, Pelletier, 2000, Liang *et al.*, 2016, Delfosse *et al.*, 2014, Yang *et al.*, 2017a).

In the current study we observed that BPA and its analogues BPB, BPF and BPS at different concentrations not only resulted in potential hazardous effects on spermatogenesis but also lead into oxidative stress in the reproductive organs of male rats by reducing the DSP and altering morphology of seminiferous tubule epithelium. The results highlight the potential toxic effect of BPA and some of its analogues in different organism tested in *in vitro* and *in vivo* studies where researcher observed the toxic effect of these compounds on male reproductive system (Zhang *et al.*, 2016, Maćczak *et al.*, 2016, Chen *et al.*, 2016b, Liang *et al.*, 2016, Ullah *et al.*, 2017, Ullah *et al.*, 2016).

CONCLUSIONS

On the basis of the results from the present study, it can be concluded that chronic exposure for a long period of time to low concentrations of BPA and its analogues BPB, BPF and BPS are capable of suppressing gonadotropins secretion from pituitary, exhibiting estrogenic and anti-androgenic effects in the mammals, inducing oxidative stress in the testicular tissue and affecting spermatogenesis by causing maturation arrest spermatogoneal stage as well as at the stage when spermatids can be seen. Further molecular studies need to be done to identify the exact mechanism of action of BPA and its analogues BPB, BPF and BPS through which it exhibits potential hazardous effects on the male reproductive tissues in mammals.

ABSTRACT

Background: Research in the past has indicated association between long term and low level of exposure of BPA in early life and neuroendocrine disorders, such as obesity, precocious puberty, diabetes, anxiety and hypertension. BPA and its analogues BPB, BPF and BPS have been reported to have similar or even more toxic effects as compared to BPA in many studies. Exposure of adult male rats resulted in decreased sperm production, testosterone secretions and histological changes in male rat testis suggesting the potential effects of BPA, BPB, BPF and BPS on sexual development in male rats.

Materials and methods: BPA and its analogues BPB, BPF and BPS different concentrations (5, 25 and 50 μg/L) in drinking water from pregnancy day 1 (PD1) to PD 21 and water was replaced by fresh water was given to pregnant female rats. Body weight and ano-genital distance (AGD), nipple retention (NR) was determined in the pups. Hormonal concentrations and histological changes were determined in testis and epididymis on PND 80.

Results: BPA and its analogues BPB, BPF and BPS different concentrations pre-natal exposed to female rats induced no significant alteration in early sexual development of male rats. Body weight gain, AGD, NR and organs weight exhibited no marked changes in the treated groups as compared to the control. Similarly, significant difference was noted in the plasma testosterone and estrogen concentrations when compared to the control. Histological parameters of both testis and epididymis revealed prominent changes in the tissues and were nearly similar to the control.

Conclusions: On the basis of the results from present study it might be concluded that BPA and its analogues BPB, BPF and BPS different concentrations exhibit marked alterations in the development of male reproductive system.

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are chemicals that alter hormone biosynthesis and these chemicals have similar structure with endogenous hormones and even exposure to them a very minute quantity can alter homeostasis. For both human and wildlife many groups of these chemicals presence in the environment having endocrine disrupting properties has led to concerns for the last few years. Wildlife exposure to these chemicals has caused disruption of sexual development and even in some fish exposure to these environmental estrogen chemicals caused feminization by males (Segner *et al.,* 2013). Estrogen play an important role in homeostasis and development of an organism and chemicals which can alter the normal estrogen signaling lead to many health effects (Kang *et al.,* 2007). Estrogenic chemicals exposure to humans have been associated with urogenital track malformations, decrease in immune function, health disease, breast and testicular cancer (Gore *et al.*, 2015).

Bisphenol A (BPA) is one of the highly produced chemicals in the world and its production has increased from approximately 5.5 million tons per year since 2010 (Metz, 2016). It is predicted that this amount is going to increase by annual rate of 5% by 2019 (Jin *et al.*, 2017). BPA is used for the manufacturing of epoxy resins and polycarbonate plastics and is found in many consumer products, including thermal papers receipt, toys, medical equipment, food and beverage containers (Im and Löffler, 2016). BPA long history of manufacturing has led to a widespread exposure in the global environment and BPA has been detected in various human bodily fluids like urine, blood and breast milk (Seachrist *et al.*, 2016, Liao *et al.*, 2012a, Vandenberg *et al.*, 2007). Human are exposed to BPA through diet, dermal contact and in inhalation of dust (Jin and Zhu, 2016, Calafat *et al.*, 2008).

BPA has estrogen like effects and its continuous exposure to human lead to many health concerns (Zhang *et al.*, 2011a, Lan *et al.*, 2017). Many reports have shown BPA to interfere with steroidogenesis and its association with serum increased heart rate and metabolic disease, reproductive disorders, diabetes and cancer (Vom Saal *et al.*, 2012, Melzer *et al.*, 2010, Dairkee *et al.*, 2008, Jin *et al.*, 2017). This has prompted the release of strict regulations on the application of BPA in infant bottles and sippy cups by government organizations (vom Saal and Myers, 2008). Consequently, several global manufacturers voluntarily phased out BPA and have started to develop various BPA alternatives, including BPB, BPF and BPS.

BPA analogues BPB, BPF and BPS have the same common structure of two hydroxyphenyl groups as BPA. These analogues are used for the manufacturing of polycarbonate plastics, paper products and phenolic resins (Yang *et al.*, 2014b, Lee *et al.*, 2013, LaFleur and Schug, 2011). However, *in vitro* studies have shown that these analogues had stronger estrogenic activities as compared to BPA (Kitamura *et al.*, 2005, Ullah *et al.*, 2018). On the other hand, BPA analogues such as BPS and BPF were capable of disrupting steroidogenesis (Kitamura *et al.*, 2005, Rosenmai *et al.*, 2014, Ullah *et al.*, 2018). Due to the increase production of these alternatives human exposure to these bisphenols is at rise which is very alarming (Liao *et al.*, 2012b). BPF and BPS have been detected in indoor dust, surface water and human urine in different population across the globe which is comparable to BPA (Liao *et al.*, 2012d, Yang *et al.*, 2014a). However, BPA analogues internal and external exposure risks remain poorly understood in the general population.

Research in the past has indicated association between long term and low level of exposure of BPA in early life and neuroendocrine disorders, such as obesity, precocious puberty, diabetes, anxiety and hypertension (Kinch *et al.*, 2015, Lang *et al.*, 2008, Thayer *et al.*, 2012, Rochester, 2013, Braun *et al.*, 2011a). Brest milk is a major energy source for infants as well as an internal source of exposure to contaminants from mother to fetus, which means that fetus and infants are more vulnerable to BPA exposure (Grob *et al.*, 2015). BPA and its analogues have been reported to have similar or even more toxic effects as compared to BPA in many studies (Chen *et al.*, 2016a, Zhang *et al.*, 2017b, Hu *et al.*, 2002, Riu *et al.*, 2011). Studies have reported that some of the BPA analogues have similar estrogenic potency as BPA (Grignard *et al.*, 2012, Masuno *et al.*, 2005). In mammals studies BPF, BPS and BPAF have been observed to have reduced testosterone, cholesterol and disruption of estrous cycle (Umano *et al.*, 2012, Feng *et al.*, 2012). These findings highlight that bisphenols marketed as safer alternatives to BPA, may similarly induce widespread and varied health effects. However, few studies have assessed the endocrine disrupting potential of these alternatives to BPA *in vivo*.

The present study was designed to understand the estrogenic mode of actions and toxicity inducing potential of BPA and its analogues BPB, BPF and BPS on sexual development of pre-natal male rats.

MATERIALS AND METHODS

Animals and treatments

A total of 65 male and 65 female Sprague Dawley male rats (150 ± 20 g were taken from Quaid-i-Azam University Animal Sciences, Primate Facility, Islamabad, Pakistan) were used in this study. All the experimental animals were kept in the facility with controlled conditions of a 12-h light and dark cycle, at 23 ± 2 °C, with relative humidity of 50 % ± 10 %, and with free access to food and water. All experimental procedures were carried out in full compliance with Quaid-i-Azam University Islamabad, Pakistan human care and laboratory animals care protocols, and approved by the experimental animals ethical committee of Quaid-i-Azam University, Islamabad, Pakistan.

Adult female rats (n=65) were placed with male rats (n=65) in the breeding cages prior to the start of the experiment. Five females and five males rats were breed in large breeding cages.

All the female rats were checked daily for the sign of pregnancy (Vaginal plug) and were kept in separated cages as the day of Pregnancy was observed. While after few days those females who had no vaginal plug were excluded from the study. The day the vaginal plug was visible was considered as gestation day 1 (GD 1). All the females were weighed daily and kept in wooden cages provided with nesting materials under standard laboratory conditions. On the GD 1 all the female rats were separated from male rats and were divided into thirteen groups containing eight female rats per group. All the animals were provided with different concentrations (5, 25 and 50 µg/L) of BPA, BPB, BPF and BPS dissolved in 0.5 % of ethanol in drinking water and control was given 0.1- 0.5 % of ethanol in drinking water (Ji *et al.*, 2013). The animals were checked daily for any signs of toxicity and the drinking water containing BPA and its analogues BPB, BPF and BPS was removed as pups were born and the day the pups were born was considered as post natal day (PND 1).

Early development study

Anu-genital distance (AGD), nipple retention (NR) and organs weight

On the day the pups were born all the pups per female were counted, weighed and sex (Male/female) was checked. Throughout the experiment the pups were observed for any sign of toxicity and weight of each group along with AGD and NR was checked on (PND 6, 14, 16, 35,

60 and 80). On PND 16, one male/per litter was euthanized and different organs (testes, epididymis, ventral prostate, seminal vesicles, bulbocavernosus muscles, bulbouretral glands, adrenals, thyroid, retroperitoneal fat pad and liver) were dissected out and weighed. All the male pups were checked for any sign of puberty from the appearance of any external sign according to (Sachs and Meisel, 1979). The day the pubertal signs were observed was noted in every group and the day puberty was more clear was noted. All the male rats were dissected on PND 80 and blood and different organs were collected for the determination of different biochemical, hormonal and histopathological parameters.

Tissue histology

Testicular tissues (Testes and epididymes) were fixed in formalin for 48 h. Dehydrated with different grades of alcohol and cleared with help of xylene the paraffin sections (5 μm) were cut and stained with hematoxylin and eosin stains to assess standard histology and morphometry according to (Ullah *et al.*, 2016). Testicular sections from 10 to 20 per group were digitized under Leica Microscope (New York Microscope company) equipped with digital camera (Canon, Japan).

For the morphometry the images were taken at 20x and 40x and the results were done with Image J software. Area of different sections was calculated with the method of (Jensen *et al.*, 2013). From 20x images 30 picture per animal were selected and known area of different area of intestinal space, epididymis tubules and seminiferous tubules was measured by the software. Number of different cell types (spermatids, spermatogonia and spermatocytes) and area was calculated and comparison of different groups with control was done.

Sperm motility and viability

Immediately after dissection, the cauda epididymis was cut slightly with a scissor in 0.5 ml pre-warmed (at 37 °C) phosphate buffered saline (PBS) (pH 7.3) containing a drop of nigrosine stain. An aliquot of 50 μL was taken, placed on a pre-cleaned and warmed (at 37 °C) glass slide and was observed under a light microscope at 40 x. A total of 100 sperm/ sample were analyzed for motility by a technician blinded to the treatment groups. Each sample was analyzed three times and the average values were used as the total sperm motility. For viability, a drop of eosin and nigrosine was added to the sperm sample. A volume of 10 μL was placed on a pre-armed and cleaned glass slide and observed under a microscope at 100 x. Ten fields were analyzed by a

person blinded to the treatment groups. A total of 100 sperm/field were checked for eosin staining and number of live and dead sperm was estimated. Each sample was repeated three times and average number was reported and expressed as percentage of live sperm.

Sperm count and daily sperm production (DSP)

DSP was done in the testicular tissues, with the help of rotostaor homogenizer (IKA-Werke, Staufen, Germany) the thawed samples were homogenized in 5 ml of solution which contained 0.5% NaCl and 5% triton X-100. The homogenized sample was diluted and samples were transpired to a neubar chamber and 19th stage spermatids were counted under microscope at 40 x. Sperm count was done in the testicular tissues as the obtained values by the sperm count in the testes were divided by 6.3 (number of days the spermatids remain in seminiferous epithelium).

Daily sperm production (DSP) = Y/6.3

Number of sperm in different parts of epididymis and sperm transient time

Immediately after dissection, the cauda epididymis was cut slightly with a scissor in 0.5 mL pre-warmed (at 37 ºC) PBS (pH 7.3) containing a drop of nigrosin stain. An aliquot of 50 µL was taken, placed on a pre-cleaned and warmed (at 37 ºC) glass slide and was observed under a light microscope at 40 x. A total of 100 sperm/sample were analyzed for motility by a technician blinded to the treatment groups. Each sample was analyzed three times and the average value was used as the total sperm motility. For viability, a drop of eosin and nigrosin was added to the sperm sample. A volume of 10 µL was placed on a pre-warmed and cleaned glass slide and observed under a microscope at 100 X. Ten fields were analyzed by a person blinded to the treatment groups. A total of 100 sperm/field were checked for eosin staining and number of live and dead sperm was estimated. Each sample was repeated three times and average number was reported and expressed as percentage of live sperm.

Hormonal Analysis

Plasma testosterone, estrogen, LH and FSH were determined by Enzymes linked immune sorbent assay (ELISA) kit purchased from Amgenix Inc.USA,

Statistical analysis

Dunnets multiple comparison tests which followed (ANOVA) was used for the comparison of different groups with control using Graph Pad Prism software. Values were expressed as Mean ± SEM and were considered significant at $P < 0.05$.

RESULTS

Effects of BPA and its analogues BPB, BPF and BPS exposure on Dams, litter size and male offspring body weights

Exposure to different concentrations of BPA and its analogues BPB, PBF and PBS during gestational period from gestational day (GD) 1 to GD 20 did not have any effect on mothers in the current study. There was no significant difference observed in the body weight gain during the pregnancy, litter size and sex ratio (Table 23). Similarly, BPA and its analogues BPB, BPF and BPS different concentrations did not show significant difference in the male pups birth weights, litter size as compared to control presented in table 23.

Effects of BPA and its analogues BPB, BPF and BPS exposure on Anu-genital distance (AGD) and Nipple retention (NR)

Anu-gental (AGD) distance and nipple retention (NR) in the different treatment groups and control is presented in table 23. There was no significant difference observed in the AGD in the male offspring after prenatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS. AGD in male from control group was 3.50 ± 0.32 mm, while in the highest concentrations exposed groups of BPA, BPB, BPF and BPS, AGD was 3.42 ± 0.08, 3.50 ± 0.02, 3.32 ± 0.11 and 3.51 ± 0.02 mm. There was little number of nipples seen in groups exposed to different concentrations of BPA, BPB, BPF and BPS and control. On the other hand, there was no significant difference observed in the number of nipple in the prenatal stage of different groups exposed to different concentrations of BPA and its analogues BPB, BPF and BPS as compared to control. Average number of nipples in control (0.24 ± 0.04) was not statistically different as compared to the BPA, BPB, BPF and BPS exposed groups presented in table 23.

Table 23. Effect of different concentrations (0, 5, 25, and 50 µg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on offspring

Treatments	Parameters					
	BW gain in Dam	Litter size	% males	Male birth weight (g)	AGD in males (mm)	NR in males
Control	89.12 ± 0.37	10.21 ± 0.37	37.03 ± 0.81	5.36 ± 0.11	3.50 ± 0.32	0.24 ± 0.04
BPA 5 ug/L	92.79 ± 0.29	10.04 ± 0.29	32.41 ± 0.50	4.81 ± 0.37	3.60 ± 0.21	0.29 ± 0.19
BPA 25 ug/L	97.39 ± 0.22	9.19 ± 0.22	31.81 ± 0.86	4.86 ± 0.16	3.16 ± 0.09	0.28 ± 0.02
BPA 50 ug/L	95.37 ± 0.31	9.31 ± 0.22	34.81 ± 1.35	4.72 ± 0.23	3.42 ± 0.08	0.30 ± 0.03
BPB 5 ug/L	93.32 ± 0.38	9.01 ± 0.31	34.34 ± 1.95	5.41 ± 0.21	3.72 ± 0.20	0.23 ± 0.01
BPB 25 ug/L	95.08 ± 0.37	9.08 ± 0.38	32.81 ± 1.39	4.81 ± 0.25	3.07 ± 0.17	0.28 ± 0.02
BPB 50 ug/L	96.64 ± 0.21	9.55 ± 0.37	33.81 ± 1.24	4.62 ± 0.15	3.50 ± 0.02	0.31 ± 0.03
BPF 5 ug/L	92.54 ± 0.39	9.53 ± 0.21	34.03 ± 1.68	5.11 ± 0.17	3.84 ± 0.25	0.28 ± 0.02
BPF 25 ug/L	93.44 ± 0.10	9.31 ± 0.39	32.34 ± 0.80	4.96 ± 0.32	3.40 ± 0.16	0.29 ± 0.03
BPF 50 ug/L	96.45 ± 0.28	9.23 ± 0.10	34.21 ± 1.35	4.46 ± 0.27	3.32 ± 0.11	0.23 ± 0.01
BPS 5 ug/L	93.08 ± 0.34	9.14 ± 0.28	32.21 ± 0.37	5.04 ± 0.15	3.58 ± 0.22	0.28 ± 0.01
BPS 25ug/L	92.88 ± 0.06	9.33 ± 0.34	32.23 ± 0.63	4.78 ± 0.24	3.24 ± 0.09	0.24 ± 0.02
BPS 50 ug/L	96.22 ± 0.06	9.46 ± 0.63	35.22 ± 2.11	4.86 ± 0.06	3.51 ± 0.02	0.27 ± 0.01

Effects of BPA and its analogues BPB, BPF and BPS exposure on the body weight and different organs weight on PND 16

Body weight in male pups was determined on Post-natal day (PND) 16 and different organs were weighed after dissection. Body weight and organs weight is represented in table 24 and 25. Prenatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS did not affect weight of reproductive organs like testes, epididymis, seminal vesicles, prostate, bulbourethral gland and bubocavernosus muscles. Similarly, non-reproductive organs like adrenals, liver and retroperitoneal fat pad were also unaffected in the groups exposed to different concentrations of BPA and its analogues BPB, BPF and BPS presented in table 24 and 25.

Effects of BPA and its analogues BPB, BPF and BPS different concentrations exposure on the puberty onset and organ weights on PND 80

All the male pups exposed to different concentrations of BPA and its analogues BPB, BPF and BPS were analyzed daily for preputial skin after post-natal day 35th. The day one of puberty was considered as the preputial skin separated in the pups. External signs of puberty analysis showed that BPA and its analogues BPB, BPF and BPS exposure did not have any effect on this parameter are presented in table 26. Body weight of male rats exposed to different treatments of BPA and its analogues BPB, BPF and BPS showed significant increase in the highest exposure groups of BPA 50 ug/L ($P < 0.05$), BPB 50 ug/L ($P < 0.05$), PBF 50 ug/L and BPS 50 ug/L ($P < 0.05$) when compared to the control. Similarly, the body weight also increased in the other treated groups of BPA and its analogues BPB, BPF and BPS but that increase was not significant to the control. All the male rats were dissected on PND 80 and different reproductive parameters were observed after prenatal exposure to different concentrations to BPA and its analogues BPB, BPF and BPS presented in table 26. There was an increase in fat pad, Liver, Kidney and adrenals but the increase was not statistically significant. Similarly, a non-significant reduction was also observed in the prostate of the exposed groups of BPA and its analogues BPB, BPF and BPS as compared to the control. On the other hand, significant increase was observed in the seminal vesicle of higher exposure groups as BPA 50 ug/L ($P < 0.05$), BPB 50 ug/L ($P < 0.05$), PBF 50 ug/L and BPS 50 ug/L ($P < 0.05$) when compared to the control presented in table 27. However, there was no significant difference observed in the other treated groups as compared to control.

Table 24. Effect of different concentrations (5, 25, and 50 µg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on organ and body weights of offspring on PND 16

Treatments	Parameters					
	Body weight (g)	Right testis (mg)	Left testis (mg)	Prostate (mg)	Epididymis (mg)	Seminal vesicle (mg)
Control	22.75 ± 0.50	63.69 ± 0.38	62.55 ± 0.74	15.81 ± 0.51	27.50 ± 1.31	8.01 ± 0.44
BPA 5 ug/L	22.97 ± 0.54	62.75 ± 0.49	61.97 ± 0.26	15.99 ± 0.51	25.68 ± 1.37	7.40 ± 0.50
BPA 25 ug/L	23.88 ± 0.70	62.59 ± 0.39	62.01 ± 0.50	17.06 ± 0.25	26.99 ± 1.22	8.41 ± 0.40
BPA 50 ug/L	24.39 ± 0.67	61.24 ± 1.46	62.59 ± 0.89	17.30 ± 0.36	24.95 ± 1.20	8.22 ± 0.37
BPB 5 ug/L	22.88 ± 0.70	61.81 ± 0.56	61.99 ± 0.27	16.68 ± 0.36	25.97 ± 1.68	7.81 ± 0.58
BPB 25 ug/L	23.02 ± 0.54	62.79 ± 0.52	62.44 ± 0.70	16.39 ± 0.39	24.64 ± 1.94	8.21 ± 0.37
BPB 50 ug/L	23.73 ± 0.63	62.06 ± 0.52	61.19 ± 1.08	16.64 ± 0.36	24.86 ± 2.07	8.42 ± 0.40
BPF 5 ug/L	24.21 ± 0.56	61.79 ± 0.56	62.99 ± 0.50	16.57 ± 0.46	25.04 ± 1.62	8.21 ± 0.37
BPF 25 ug/L	23.50 ± 0.74	62.37 ± 1.33	62.57 ± 0.50	16.86 ± 0.25	26.10 ± 1.58	8.01 ± 0.31
BPF 50 ug/L	23.19 ± 0.74	61.82 ± 0.03	60.43 ± 1.14	16.97 ± 0.40	25.66 ± 1.82	8.81 ± 0.20
BPS 5 ug/L	22.90 ± 0.43	62.61 ± 0.77	62.21 ± 0.64	16.72 ± 0.36	24.59 ± 1.86	8.41 ± 0.40
BPS 25ug/L	23.61 ± 0.58	62.41 ± 0.35	62.55 ± 0.51	16.86 ± 0.25	25.95 ± 1.62	8.40 ± 0.24
BPS 50 ug/L	23.44 ± 0.76	61.61 ± 0.37	60.55 ± 0.57	16.72 ± 0.37	25.85 ± 1.68	8.40 ± 0.24

Table 25. Effect of different concentrations (5, 25, and 50 µg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on organ of offspring on PND 16

Treatments	Parameters				
	Bulbourethral gland (mg)	Adrenals (mg)	Bulbocavernosus muscles (mg)	Fat pad (mg)	Liver (mg)
Control	1.86 ± 0.05	7.48 ± 0.71	33.52 ± 0.72	41.81 ± 1.05	743.70 ± 84.56
BPA 5 ug/L	1.66 ± 0.11	8.36 ± 0.35	33.99 ± 0.98	41.74 ± 1.36	783.59 ± 61.95
BPA 25 ug/L	1.61 ± 0.05	7.84 ± 0.38	33.52 ± 0.72	44.57 ± 1.49	666.92 ± 102.32
BPA 50 ug/L	1.66 ± 0.40	8.46 ± 0.31	31.84 ± 0.40	43.32 ± 1.43	680.12 ± 81.72
BPB 5 ug/L	1.66 ± 0.05	8.64 ± 0.57	32.46 ± 0.72	42.13 ± 2.13	616.18 ± 52.58
BPB 25 ug/L	1.64 ± 0.13	8.28 ± 0.59	34.81 ± 1.30	43.80 ± 1.13	696.92 ± 104.11
BPB 50 ug/L	1.68 ± 0.05	8.75 ± 0.32	34.48 ± 1.40	42.63 ± 1.23	805.41 ± 62.45
BPF 5 ug/L	1.66 ± 0.05	7.87 ± 0.41	34.24 ± 1.62	42.31 ± 1.80	622.59 ± 86.60
BPF 25 ug/L	1.65 ± 0.09	8.06 ± 0.23	33.37 ± 1.71	45.72 ± 1.04	747.34 ± 82.36
BPF 50 ug/L	1.74 ± 0.02	8.90 ± 0.28	32.88 ± 0.95	43.01 ± 2.15	554.38 ± 44.29
BPS 5 ug/L	1.62 ± 0.11	8.66 ± 0.70	32.99 ± 0.79	42.13 ± 2.13	620.36 ± 79.52
BPS 25ug/L	1.70 ± 0.08	8.46 ± 0.42	34.86 ± 1.21	43.38 ± 1.03	709.32 ± 75.85
BPS 50 ug/L	1.61 ± 0.09	8.63 ± 0.98	34.71 ± 1.29	43.17 ± 1.50	721.72 ± 98.61

Table 26. Effect of different concentrations (5, 25, and 50 µg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on organ and body weights of offspring on PND 80

Treatments	Parameters					
	Puberty onset (day)	Body weight (g)	Left testis (g)	Right testis (g)	Left epididymis (g)	Right epididymis (g)
Control	43.41 ± 0.52	192.26 ± 0.70	1.14 ± 0.01	1.16 ± 0.05	0.49 ± 0.03	0.52 ± 0.03
BPA 5 ug/L	44.13 ± 0.85	192.73 ± 0.71	1.14 ± 0.01	1.13 ± 0.09	0.44 ± 0.05	0.48 ± 0.05
BPA 25 ug/L	43.37 ± 0.72	203.06 ± 1.15	1.12 ± 0.10	1.14 ± 0.01	0.46 ± 0.04	0.46 ± 0.02
BPA 50 ug/L	42.59 ± 0.85	210.30 ± 3.73*	1.14 ± 0.01	1.17 ± 0.07	0.44 ± 0.01	0.52 ± 0.03
BPB 5 ug/L	43.46 ± 0.63	191.68 ± 3.26	1.13 ± 0.06	1.14 ± 0.07	0.42 ± 0.05	0.48 ± 0.02
BPB 25 ug/L	43.50 ± 0.45	204.55 ± 5.89	1.14 ± 0.01	1.17 ± 0.05	0.44 ± 0.01	0.57 ± 0.04
BPB 50 ug/L	42.62 ± 0.69	211.35 ± 8.01*	1.14 ± 0.06	1.14 ± 0.03	0.45 ± 0.01	0.47 ± 0.02
BPF 5 ug/L	42.75 ± 0.28	190.35 ± 3.16	1.13 ± 0.01	1.15 ± 0.01	0.44 ± 0.08	0.48 ± 0.04
BPF 25 ug/L	43.28 ± 0.44	204.35 ± 5.09	1.13 ± 0.08	1.13 ± 0.08	0.44 ± 0.01	0.55 ± 0.02
BPF 50 ug/L	42.42 ± 0.37	210.33 ± 8.71*	1.15 ± 0.08	1.17 ± 0.06	0.44 ± 0.02	0.51 ± 0.04
BPS 5 ug/L	43.06 ± 0.85	204.35 ± 3.98	1.13 ± 0.09	1.15 ± 0.06	0.44 ± 0.01	0.42 ± 0.02
BPS 25ug/L	43.55 ± 0.58	200.37 ± 7.09	1.12 ± 0.09	1.16 ± 0.07	0.44 ± 0.08	0.46 ± 0.03
BPS 50 ug/L	42.10 ± 0.59	210.41 ± 6.31*	1.14 ± 0.01	1.16 ± 0.08	0.45 ± 0.09	0.45 ± 0.03

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

Table 27. Effect of different concentrations (5, 25, and 50 µg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on organ and body weights of offspring on PND 80

Treatments	Parameters					
	Seminal vesicle weight (g)	Prostate weight (g)	Fat pad weight (g)	Kidney weight (g)	Liver weight (g)	Adrenals weight (mg)
Control	1.17 ± 0.01	0.53 ± 0.05	1.38 ± 0.01	0.91 ± 0.02	5.90 ± 0.20	35.04 ± 0.76
BPA 5 ug/L	1.16 ± 0.08	0.43 ± 0.04	1.34 ± 0.02	0.93 ± 0.05	4.88 ± 0.25	35.08 ± 0.50
BPA 25 ug/L	1.14 ± 0.07	0.59 ± 0.03	1.44 ± 0.03	0.94 ± 0.05	5.67 ± 0.21	36.26 ± 0.21
BPA 50 ug/L	1.11 ±0.02*	0.43 ± 0.01	1.48 ± 0.02	0.91 ± 0.02	5.93 ± 0.22	36.72 ± 0.20
BPB 5 ug/L	1.16 ± 0.09	0.54 ± 0.01	1.35 ± 0.04	0.91 ± 0.05	6.13 ± 0.25	34.88 ± 0.51
BPB 25 ug/L	1.16 ± 0.01	0.50 ± 0.07	1.45 ± 0.03	0.94 ± 0.07	6.41 ± 0.03	36.17 ± 0.28
BPB 50 ug/L	1.13 ± 0.01*	0.45 ± 0.02	1.46 ± 0.03	0.91 ± 0.01	6.45 ± 0.17	36.52 ± 0.31
BPF 5 ug/L	1.14 ± 0.04	0.57 ± 0.04	1.35 ± 0.02	0.93 ± 0.02	6.09 ± 0.22	34.68 ± 0.38
BPF 25 ug/L	1.13 ± 0.07	0.48 ± 0.02	1.45 ± 0.02	0.95 ± 0.01	6.43 ± 0.03	36.52 ± 0.08
BPF 50 ug/L	1.13 ± 0.07*	0.44 ± 0.01	1.46 ± 0.02	0.92 ± 0.03	6.67 ± 0.05	36.52 ± 0.54
BPS 5 ug/L	1.16 ± 0.01	0.54 ± 0.02	1.44 ± 0.01	0.92 ± 0.01	6.06 ± 0.26	34.68 ± 0.66
BPS 25ug/L	1.14 ± 0.02	0.47 ± 0.04	1.47 ± 0.03	0.94 ± 0.02	6.41 ± 0.03	35.86 ± 0.52
BPS 50 ug/L	1.13 ± 0.06*	0.48 ± 0.03	1.44 ± 0.03	0.91 ± 0.03	6.12 ± 0.22	36.08 ± 0.53

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

Effects of BPA and its analogues BPB, BPF and BPS different concentration exposure on DSP and number of sperm in different parts of epididymis

Results of DSP in the control and exposed groups of BPA and its analogues BPB, BPF and BPS presented in table 28. There were some alterations observed in some of the parameters of DSP after exposure to different concentrations of BPA and its analogues BPB, BPF and BPS. Significant reduction was observed in the DSP of higher exposure groups of BPA 50 ug/L ($P < 0.05$), BPB 50 ug/L ($P < 0.05$), PBF 50 ug/L and BPS 50 ug/L ($P < 0.05$) when compared to the control. Sperm number in the caput/carpus region of epididymis in the exposed groups was comparable to the control as significant reduction was observed in BPA 25 ug/L ($P < 0.05$) and BPA 50 ug/L ($P < 0.05$) when compared to the control. Number of sperm in the caput/carpus region was reduced significantly ($P < 0.05$) in BPB 25 and 50 ug/L treated groups as compared to the control. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose levels of 25 and 50 ug/L in comparison to the control group. On the other hand, BPS 25 and 50 ug/L significantly reduced ($P < 0.05$) sperm number in the caput/carpus region of epididymis; however, the low doses groups did not reduce number of sperm in both caput and carpus region as compared to the control (Table 28).

Moreover, sperm transit time in the caput/carpus epididymis, cauda epididymis sperm number and sperm transit time in the cauda epididymis was not statistically different in the exposed groups as compared to the control group. This means that maternal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS affects sperm number and sperm transit time in cauda epididymis of the rats on PND 80 (Table 28).

Table 28. Effect of different concentrations (5, 25, and 50 µg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PD 1 on daily sperm production (DSP) and sperm in different parts of epididymis of rats on postnatal day (PND 80)

Treatments	Parameters				
	DSP × 106	Caput/carpus epididymis sperm number (×106/g organ)	Sperm transit time in the caput/corpus epididymis (days)	Cauda epididymis sperm number (× 106/g organ)	Sperm transit time in the cauda epididymis (days)
Control	73.37±0.6	303.17 ± 1.39	4.13 ± 0.03	471.16 ± 9.47	6.63 ± 0.07
BPA 5 ug/L	62.28±0.3	296.68 ± 3.92	4.19 ± 0.05	465.52 ± 9.63	6.52 ± 0.05
BPA 25 ug/L	62.50±2.1	291.72 ± 2.05*	4.15 ± 0.05	465.15 ± 2.97	6.40 ± 0.04
BPA 50 ug/L	61.46±0.9*	301.83 ± 4.94*	4.28 ± 0.02	462.90 ± 4.49	6.51 ± 0.08
BPB 5 ug/L	63.37±0.7	295.08 ± 2.13	4.26 ± 0.02	466.28 ± 6.88	6.45 ± 0.02
BPB 25 ug/L	62.48±0.7	293.91 ± 2.06*	4.21 ± 0.03	464.15 ± 2.56	6.43 ± 0.03
BPB 50 ug/L	61.32±1.8*	293.92 ± 1.53*	4.23 ± 0.03	462.58 ± 4.63	6.52 ± 0.10
BPF 5 ug/L	64.17±0.5	295.19 ± 2.07	4.29 ± 0.07	466.76 ± 3.67	6.43 ± 0.03
BPF 25 ug/L	63.61±1.9	294.03 ± 1.04*	4.31 ± 0.07	463.55 ± 2.02	6.47 ± 0.03
BPF 50 ug/L	61.52±0.6*	293.01 ± 2.35*	4.23 ± 0.03	461.15 ± 4.77	6.57 ± 0.09
BPS 5 ug/L	63.28±1.5	295.57 ± 1.57	4.21 ± 0.03	464.48 ± 6.16	6.47 ± 0.03
BPS 25ug/L	62.33±0.2	293.43 ± 1.79*	4.17 ± 0.02	465.95 ± 3.18	6.53 ± 0.01
BPS 50 ug/L	61.26±0.6*	293.19 ± 1.92*	4.18 ± 0.03	462.30 ± 5.94	6.50 ± 0.11

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

Effects of BPA and its analogues BPB, BPF and BPS different concentrations exposure on histopathology of testis in male rats

Histopathological results about testis in different treatment groups of BPA and its analogues BPB, BPF and BPS and control are presented in table 29 and fig 14.

On PND 80 prenatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS in the area % of seminiferous tubule exhibited marked changes in the testis histology. Significant reduction was observed in the area of seminiferous tubules in BPA 25 ug/L ($P < 0.05$) and BPA 50 ug/L ($P < 0.001$) when compared to control. Similarly, there was also significant reduction observed in the area % of seminiferous tubules BPB 25 ug/L ($P < 0.05$) and BPB 50 ug/L ($P < 0.01$) when compared to the control. Significant reduction was also observed in the area of seminiferous tubules in BPF 25 ug/L ($P < 0.01$) and BPB 50 ug/L ($P < 0.001$) when compared to control. Similarly, BPS 25 ug/L and BPS 50 caused significant reduction in the area of seminiferous tubules as compared to control. On the other hand, there was no significant reduction observed in the lower concentrations of BPA and its analogues BPB, BPF and BPS as compared to control group. Moreover, marked changes were also observed in the area % of interstitial space on PND 80 in different concentrations of BPA and its analogues BPB, BPF and BPS in the histology of testis. Significant reduction was observed in the BPA 50 ug/L ($P < 0.05$) when compared to the control. Area % of interstitial space reduced significantly ($P < 0.05$) in BPB 50 ug/L when compared to the control. Similarly, BPF 5 ug/L treatment caused significant reduction ($P < 0.05$) when compared to control. On the other hand, BPS 5 ug/L also caused significantly reduction ($P < 0.05$) in the area % of interstitial space; however, other doses did not reduce area % of interstitial space as compared to the control. Significant reduction was observed in the area of lumen of BPA 50 ug/L ($P < 0.001$) when compared to control. The % area of lumen reduced significantly ($P < 0.001$) in BPB 50 ug/L when compared to the control. Similarly, BPF 50 ug/L treatment caused significant reduction ($P < 0.001$) when compared to control. Significant reduction was also observed in the treated group of BPS 50 ug/L ($P < 0.001$) area of lumen as compared to control. However, there was no significant difference observed in the area of lumen in treated groups with BPA and its analogues BPB, BPF and BPS 5 and 25 ug/L as compared to control.

On the PND 80 prenatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS did not cause significant difference in the area % of epithelium in the testis

histology. Seminiferous tubules diameter in different treatment groups and control is presented in table 30. Significant reduction was observed in BPA 50 ug/L ($P < 0.05$) when compared to the control. Seminiferous tubules diameter was also reduced significantly ($P < 0.05$) in BPB 50 ug/L treated group. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose level of 50 ug/L in comparison to the control. On the other hand, PBS 50 ug/L significantly reduced ($P < 0.05$) seminiferous tubule diameter. However, 5ug/L doses of BPB and its analogues BPB, BPF and BPS did not reduce seminiferous tubule diameter as compared to control.

Seminiferous tubule epithelial height in the testis histology showed significant increase BPA 50 ug/L ($P < 0.05$) when compared to control. Epithelial height in the seminiferous tubules was increased significantly ($P < 0.01$) in BPB 50 ug/L treated group in comparison to the control group. Similarly, BPF treatment caused significant increase ($P < 0.05$) at dose level of 50 ug/L. However, BPS 50 ug/L significantly increased ($P < 0.01$) seminiferous tubules epithelial height as compared to the control group.

Caput and cauda epididymis histology of BPA and its analogues BPB, BPF and BPS after PND 80 of exposure

On PND 80, prenatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS exhibited no marked changes in the caput epididymis histology is presented in table 30 and fig 15. Histology of caput epididymis was evaluated and diameter of lumen and tubules, height of epithelial, lumen and epithelial percentage were not significantly different in the exposed groups as compared to the control.

Cauda epididymis histology of different treatment groups and control is presented in table no 31 and fig 16. There was no difference observed in the area covered by epithelium and lumen in the histology of cauda epididymis. Maternal exposure to different doses of BPA and its analogues BPB, BPF and BPS did not have any significant difference in the tubular diameter, lumen diameter and epithelial height after exposure.

Table 29. Effect of different concentrations (5, 25, and 50 µg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on testis histology of rats on PND 80

Treatments	Parameters					
	Area % of seminiferous tubule	Area % of interstitial space	% area of lumen	% area of epithelium	Seminiferous tubule diameter (µm)	Seminiferous tubule epithelial height (µm)
Control	88.12 ± 0.4	14.17 ± 0.5	15.65 ± 0.1	84.56 ± 0.3	226.32 ± 2.8	59.03 ± 0.2
BPA 5 ug/L	87.07 ± 1.2	13.21 ± 0.5	15.60 ± 0.2	84.50 ± 0.5	226.21 ± 0.3	57.72 ± 0.3
BPA 25 ug/L	85.10 ± 0.5*	12.32 ± 0.4	15.35 ± 0.4	84.70 ± 0.4	223.97 ± 0.3	58.28 ± 0.5
BPA 50 ug/L	84.06 ± 0.6***	11.89 ± 0.2*	15.01 ± 0.3***	84.74 ± 0.6	221.94 ± 0.6*	60.79 ± 0.1*
BPB 5 ug/L	86.21 ± 0.4	13.90 ± 0.5	15.76 ± 0.1	84.52 ± 0.4	226.64 ± 0.3	58.48 ± 0.3
BPB 25 ug/L	85.08 ± 0.3*	12.99 ± 0.3	15.48 ± 0.5	84.64 ± 0.4	223.75 ± 0.2	58.32 ± 0.3
BPB 50 ug/L	84.50 ± 0.6**	11.99 ± 0.6*	15.04 ± 0.2***	84.77 ± 0.3	222.92 ± 0.6*	61.05 ± 0.3**
BPF 5 ug/L	87.61 ± 0.6	13.57 ± 0.3	15.58 ± 0.1	84.56 ± 0.4	225.41 ± 0.8	57.90 ± 0.4
BPF 25 ug/L	84.61 ± 0.9**	12.77 ± 0.4	15.45 ± 0.4	84.74 ± 0.8	224.37 ± 0.4	58.74 ± 0.3
BPF 50 ug/L	83.90 ± 0.6***	11.99 ± 0.5*	15.02 ± 0.3***	84.74 ± 0.6	223.52 ± 0.8*	60.85 ± 0.5*
BPS 5 ug/L	86.37 ± 0.2	13.17 ± 0.3	15.67 ± 0.1	84.54 ± 0.8	226.24 ± 0.3	58.47 ± 0.1
BPS 25ug/L	84.74 ± 0.5**	12.95 ± 0.5	15.37 ± 0.3	84.72 ± 0.7	224.17 ± 0.4	58.09 ± 0.6
BPS 50 ug/L	83.86 ±0.5***	12.15 ± 0.2*	15.04 ± 0.1***	84.72 ± 0.1	222.32 ± 0.5*	61.14 ± 0.3**

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

***: Indicate significance at $p < 0.001$ vs control

Table 30. Effect of different concentrations (5, 25, and 50 µg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on caput epididymis histology of rats on PND 80

Treatments	Parameters				
	Tubular diameter (µm)	Lumen diameter (µm)	Epithelial height (µm)	Epithelium (%)	Lumen (%)
Control	332.40 ± 1.63	246.01 ± 3.60	26.01 ± 2.25	33.25 ± 2.39	70.75 ± 2.65
BPA 5 ug/L	330.81 ± 1.80	240.60 ± 2.63	25.41 ± 1.91	32.01 ± 1.51	69.71 ± 1.96
BPA 25 ug/L	330.21 ± 2.26	238.10 ± 1.64	24.10 ± 0.77	31.51 ± 0.50	68.50 ± 2.03
BPA 50 ug/L	329.20 ± 1.52	237.20 ± 1.01	23.81 ± 0.86	29.25 ± 2.51	64.25 ± 2.89
BPB 5 ug/L	329.01 ± 2.09	240.60 ± 3.31	25.21 ± 1.46	32.93 ± 1.08	69.51 ± 4.39
BPB 25 ug/L	328.43 ± 1.20	238.20 ± 1.49	25.60 ± 1.96	31.65 ± 0.49	68.75 ± 4.37
BPB 50 ug/L	329.23 ± 1.01	235.80 ± 2.21	22.75 ± 0.91	29.16 ± 1.14	65.70 ± 2.81
BPF 5 ug/L	328.45 ± 0.74	240.81 ± 1.98	25.61 ± 1.72	32.65 ± 2.19	67.91 ± 1.72
BPF 25 ug/L	331.21 ± 2.51	239.80 ± 2.48	24.80 ± 1.39	31.05 ± 1.85	65.25 ± 1.01
BPF 50 ug/L	328.81 ± 1.77	237.20 ± 1.49	23.81 ± 1.38	29.21 ± 1.15	64.31 ± 3.26
BPS 5 ug/L	329.60 ± 1.02	240.60 ± 2.83	25.60 ± 0.87	33.42 ± 1.60	68.91 ± 1.43
BPS 25ug/L	329.82 ± 0.58	239.80 ± 2.99	24.01 ± 1.48	30.50 ± 2.42	66.05 ± 0.73
BPS 50 ug/L	329.41 ± 1.24	237.81 ± 2.88	23.31 ± 0.87	28.01 ± 1.51	65.71 ± 1.61

Table 31. Effect of different concentrations (5, 25 and 50 µg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on cauda epididymis histology of rats on PND 80

Treatments	Parameters				
	Tubular diameter (µm)	Lumen diameter (µm)	Epithelial height (µm)	Epithelium (%)	Lumen (%)
Control	442.41 ± 1.43	414.81 ± 2.17	28.65 ± 1.06	32.85 ± 2.89	66.95 ± 2.05
BPA 5 ug/L	439.62 ± 1.42	411.81 ± 1.11	27.51 ± 1.47	31.10 ± 2.26	67.33 ± 1.08
BPA 25 ug/L	439.41 ± 4.69	410.22 ± 2.71	26.71 ± 0.87	28.52 ± 0.83	66.52 ± 1.98
BPA 50 ug/L	439.01 ± 2.73	409.21 ± 2.42	26.22 ± 1.77	26.95 ± 1.83	69.25 ± 2.36
BPB 5 ug/L	438.60 ± 1.63	412.64 ± 1.62	27.61 ± 1.46	28.71 ± 7.13	67.51 ± 2.50
BPB 25 ug/L	439.61 ± 3.04	414.81 ± 2.39	26.12 ± 1.70	26.45 ± 1.25	67.95 ± 2.05
BPB 50 ug/L	439.12 ± 3.47	413.82 ± 2.10	25.61 ± 2.03	25.95 ± 1.46	69.65 ± 1.72
BPF 5 ug/L	438.83 ± 0.69	413.61 ± 0.93	27.82 ± 2.44	30.71 ± 2.62	67.91 ± 2.43
BPF 25 ug/L	438.61 ± 1.43	412.40 ± 1.69	26.81 ± 2.41	29.55 ± 2.42	69.45 ± 2.02
BPF 50 ug/L	449.01 ± 1.21	412.21 ± 1.77	26.21 ± 1.01	27.11 ± 6.44	69.71 ± 3.90
BPS 5 ug/L	439.41 ± 2.15	413.22 ± 4.32	27.21 ± 2.21	29.11 ± 2.58	67.73 ± 2.58
BPS 25ug/L	439.03 ± 1.37	413.21 ± 1.58	26.81 ± 3.13	27.35 ± 1.35	68.05 ± 1.89
BPS 50 ug/L	439.82 ± 1.59	412.61 ± 1.63	25.62 ± 3.28	27.11 ± 1.34	69.51 ± 4.00

Table 32: Effect of different concentrations (5, 25, and 50 µg/L) of BPA and its analogies BPB, BPF and BPS exposure from GD 1 to PD 1 on plasma testosterone and estrogen concentrations in rats on PD 80

Treatments	Parameters			
	Testosterone (ng/ml)	Estradiol (pg/ml)	LH (ng/ml)	FSH (mIU/ml)
Control	4.82 ± 0.04	1.27 ± 0.09	1.67 ± 0.06	1.41 ± 0.19
BPA 5 ug/L	4.48 ± 0.35	1.24 ± 0.08	1.56 ± 0.06	0.91 ± 0.18
BPA 25 ug/L	4.41 ± 0.14	2.12 ± 0.40	1.45 ± 0.04	0.93 ± 0.18
BPA 50 ug/L	3.36 ± 0.33*	3.60 ± 0.38***	1.16 ± 0.03***	0.57 ± 0.05**
BPB 5 ug/L	4.45 ± 0.13	1.87 ± 0.39	1.54 ± 0.04	0.92 ± 0.23
BPB 25 ug/L	4.31 ± 0.32	2.01 ± 0.41	1.49 ± 0.04	0.97 ± 0.22
BPB 50 ug/L	3.36 ± 0.30*	3.55 ± 0.40***	1.18 ± 0.02***	0.54 ± 0.02**
BPF 5 ug/L	4.29 ± 0.12	1.33 ± 0.03	1.53 ± 0.09	0.91 ± 0.15
BPF 25 ug/L	4.03 ± 0.19	2.38 ± 0.32	1.48 ± 0.06	0.94 ± 0.17
BPF 50 ug/L	3.30 ± 0.46*	3.28 ± 0.38***	1.21 ± 0.03***	0.55 ± 0.03**
BPS 5 ug/L	4.39 ± 0.55	1.43 ± 0.03	1.57 ± 0.08	0.90 ± 0.14
BPS 25ug/L	3.71 ± 0.28	2.54 ± 0.48	1.46 ± 0.03	0.96 ± 0.24
BPS 50 ug/L	3.45 ± 0.43*	3.79 ± 0.27***	1.21 ± 0.02***	0.53 ± 0.05**

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

***: Indicate significance at $p < 0.001$ vs control

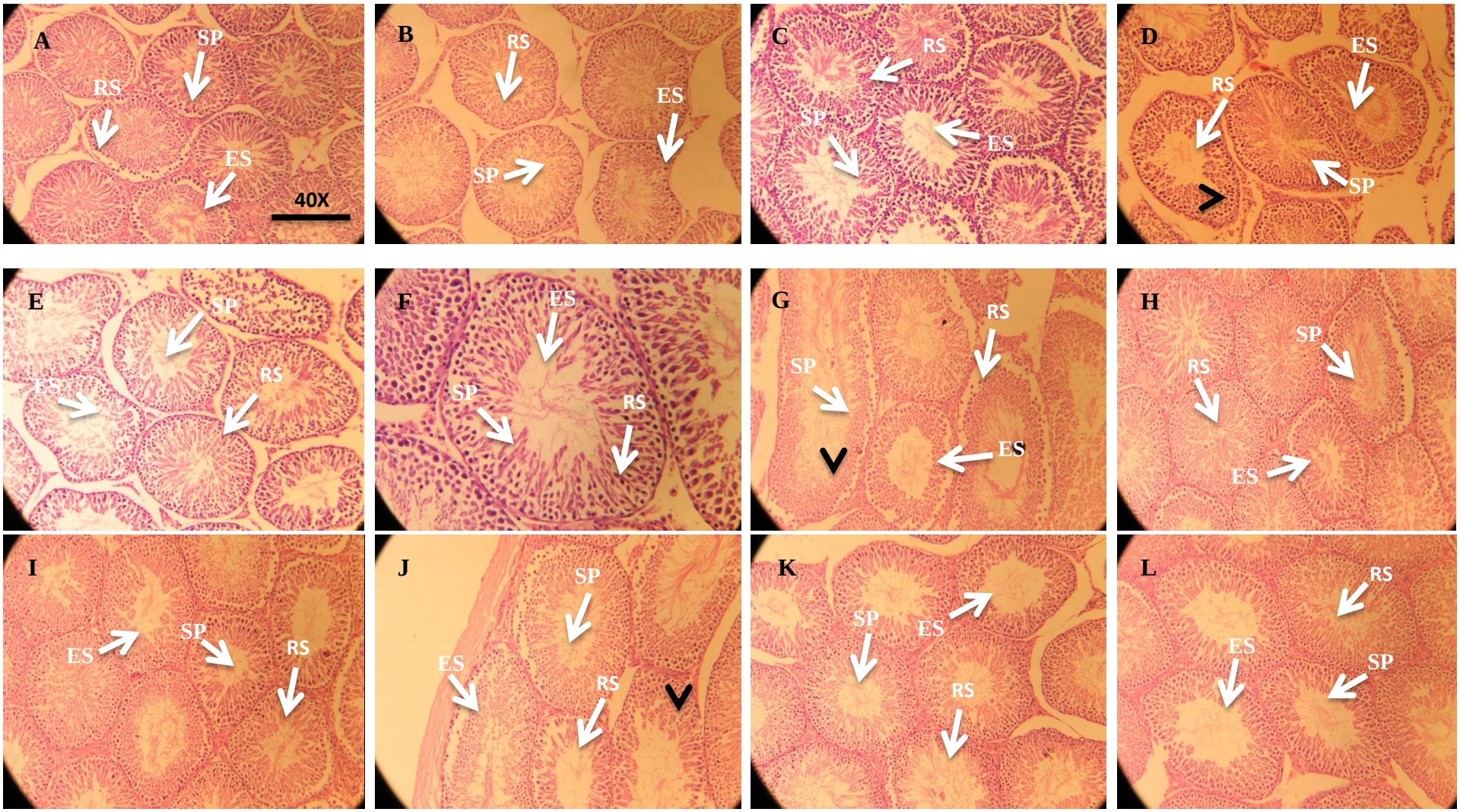
Effects of different concentrations (5, 25 and 50 µg/L) of BPA and its analogues BPB, BPF and BPS exposure from GD 1 to PND 1 on plasma testosterone, estradiol, LH and FSH concentrations in male rats on PND 80

Plasma testosterone concentrations in different treatment groups and control are presented in table 32. Significant reduction was observed in BPA 50 ug/L ($P < 0.05$) when compared to the control. Testosterone concentrations reduced significantly in BPB 50 ug/L ($P < 0.05$) when compared to control. Testosterone concentrations reduced significantly ($P < 0.05$) in BPF 50 ug/L treated groups as compared to the control. On the other hand, BPS 50 ug/L significantly reduced ($P < 0.05$) testosterone in plasma; however, other doses did not reduce plasma testosterone as compared to the control.

Plasma estradiol after PND 80 of exposure showed significant increase in BPA 50 ug/L ($P < 0.001$) as compared to control. BPB 50 ug/L caused significant increase ($P < 0.001$) in plasma estradiol as compared to control. Similarly, BPF 50 ug/L treatment caused significant increase ($P < 0.001$) in the estradiol of treated group as compared to the control. On the other hand, BPS 50 ug/L significantly increased ($P < 0.001$) estradiol in plasma; however, other doses 5 and 25 ug/L did not reduce plasma estradiol as compared to the control.

Plasma LH concentrations in different treatment groups and control are presented in presented in table 32. Significant reduction was observed in BPA 50 ug/L ($P < 0.001$) when compared to control. LH concentration was reduced significantly ($P < 0.001$) in BPB 50 ug/L treated group as compared to control. Similarly, BPF treatment caused significant reduction ($P < 0.001$) at dose level of 50 ug/L. However, BPF 5 and 25 ug/L did not affect testosterone concentrations significantly. On the other hand, BPS 50 ug/L significantly reduced ($P < 0.001$) LH in plasma; However, there was no significant difference observed in the other treated groups of BPA and its analogues BPB, BPF and BPS when compared to control.

Plasma FSH concentrations in the different treated groups of BPA and its analogues BPB, BPF and BPS are presented in table 32. Significant reduction in BPA, BPB, BPF and BPS 50 ug/L ($P < 0.01$) was observed when compared to the control. However, BPA, BPB, BPF and BPS 5 and 25 ug/L did not affect plasma FSH concentrations as compared to the control.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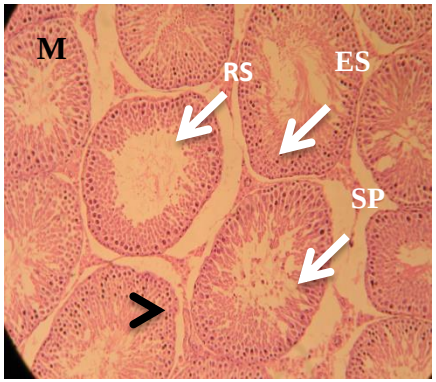

Fig 14: Photomicrograph from testicular tissue showing (A) control; having thick epithelium with normal spermatogonia (SP), Round spermatids (RS), Elongated spermatids (ES) and filled lumen with sperm (B, C and D); BPA (5, 25 and 50 µg/L) treatment presenting seminiferous tubules with epithelium (Line without arrow head) and spermatids (White arrow); (E, F and G) BPB (5, 25 and 50 µg/L) treatment presenting seminiferous tubules with epithelium (Line without arrow head) and elongating spermatids (White arrow); (H, I and J) BPF (5, 25 and 50 µg/L) treatment presenting seminiferous tubules with epithelium (Line without arrow head) and elongating spermatids (White arrow); (K, L and M) BPS (5, 25 and 50 µg/L) treatment presenting seminiferous tubules with epithelium (Line without arrow head) and spermatids (White arrow). H&E (40x).

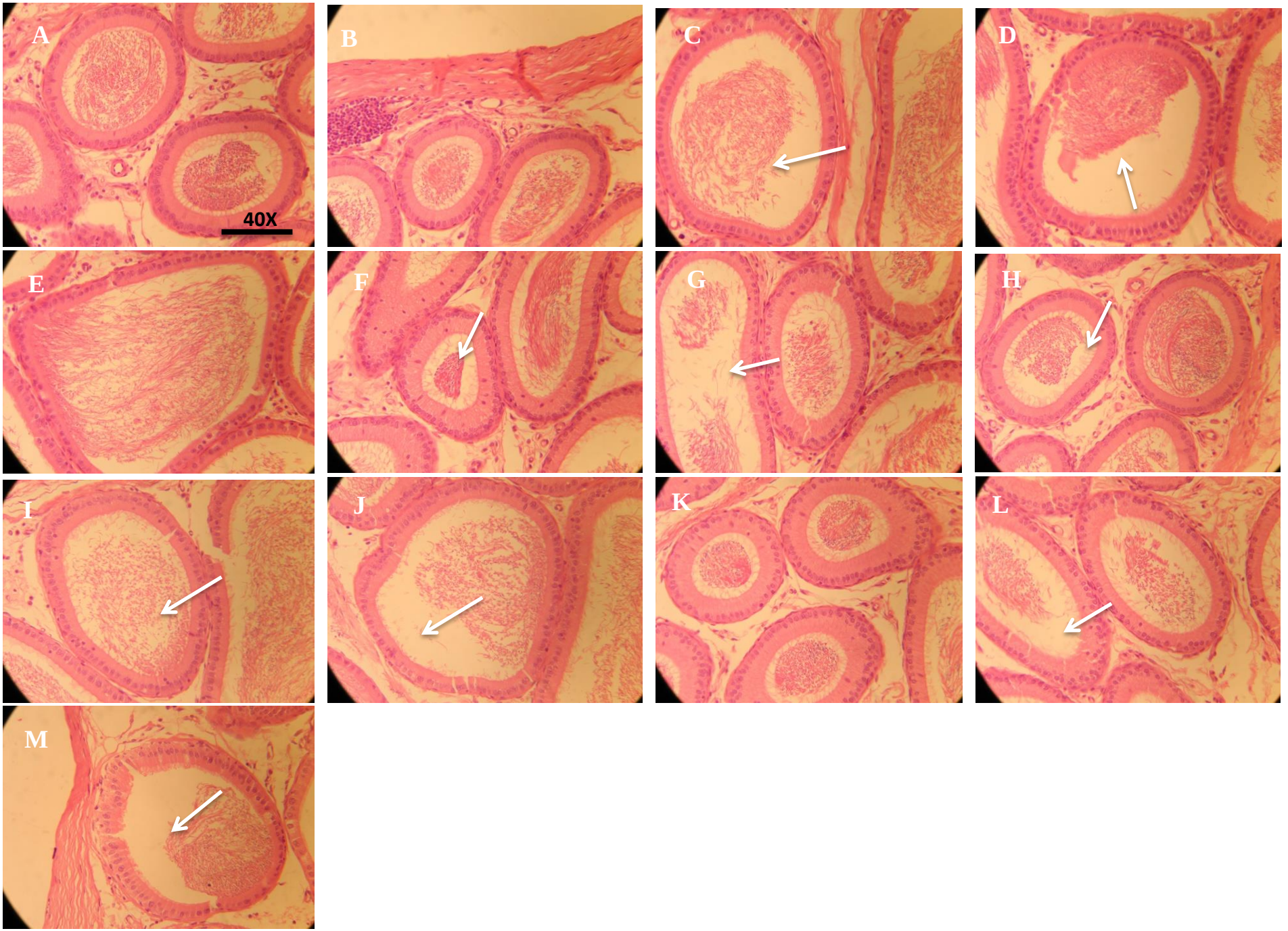
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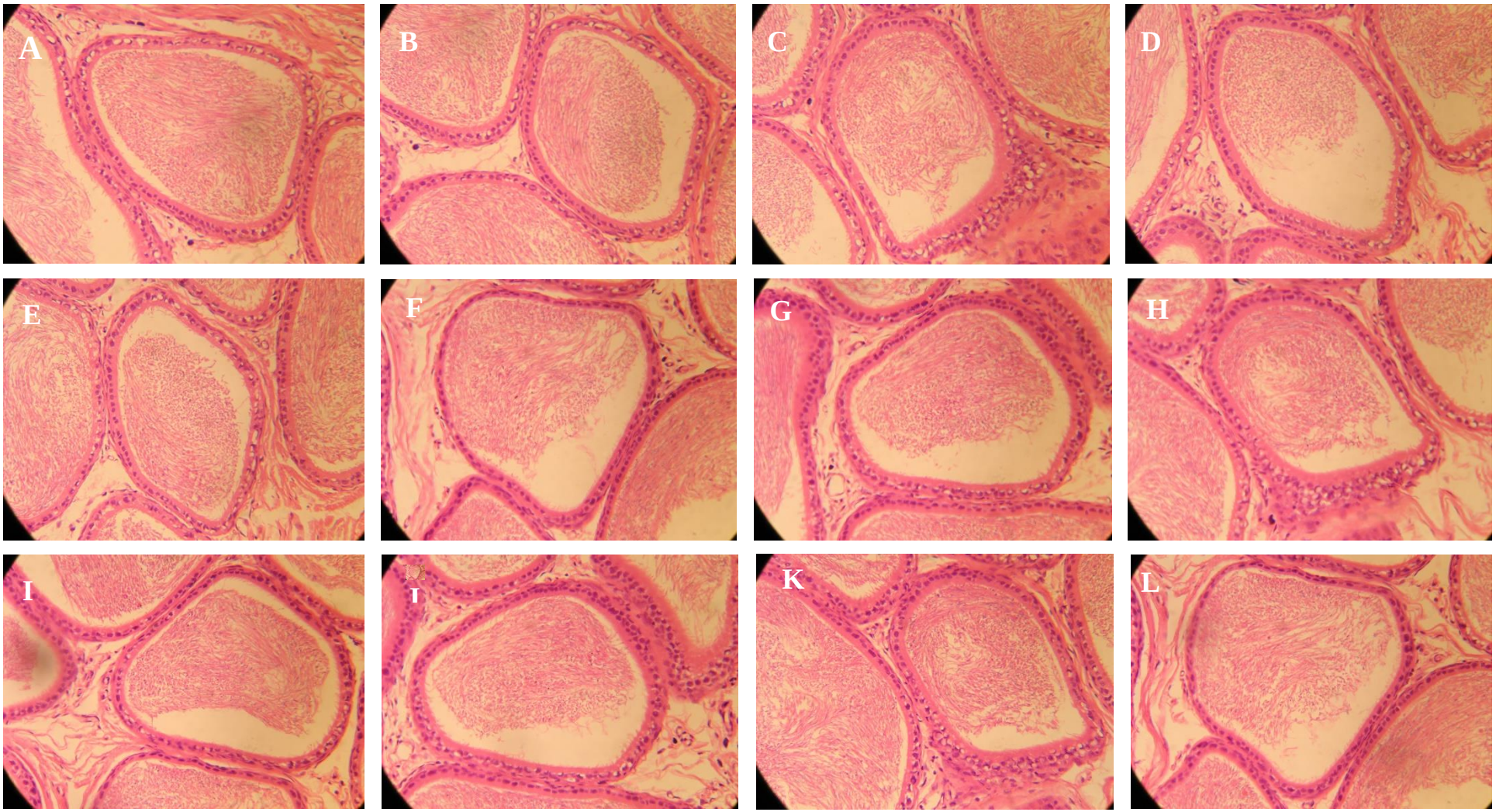
Fig 15: Photomicrograph of caput epididymis tissue showing (A) control; with compact arrangement of caput tubules with sperm filled lumen (B) BPA (5 µg/L) exposed group, presenting normal caput tubules like in the control (C), BPA (25 µg/L) exposed group showing seminiferous tubules with less number of sperm in the lumen (Arrow) and (D) BPA (50 µg/L) exposed group presenting caput tubules with empty lumen (Arrow). Similarly, (E) BPB (5 µg/L) exposed group, presenting normal caput tubules, (F) BPB (25 µg/L) exposed group showing less number of sperms in the lumen, (G) BPB (50 µg/L) exposed group showing less number of sperms and empty lumen (Arrow). (H) BPF (5 µg/L) exposed group, presenting normal caput tubules, (I) (25 µg/L) exposed group showing seminiferous tubules with less number of sperm in the lumen (Arrow) and (J) BPF (50 µg/L) exposed group showing less number of sperms and empty lumen (Arrow). K, L BPS (5 and 25 µg/L) exposed groups showing caput tubules with less number of sperms in the lumen and (M) BPS (50 µg/L) exposed group presenting less number of sperms and empty lumen. H&E (40x).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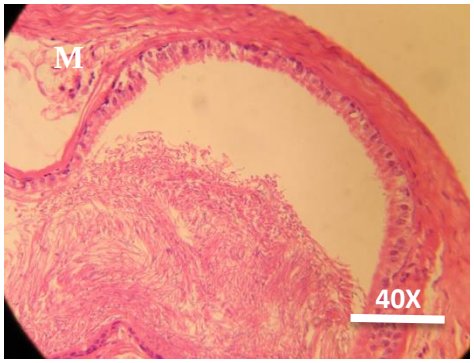

Fig 16: Photomicrograph of cauda epididymis tissue showing (A) control; with compact arrangement of cauda tubules with sperm filled lumen (B) BPA (5 µg/L) exposed group, presenting normal caput tubules like in the control (C) BPA (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (D) BPA (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. Similarly, (E) BPB (5 µg/L) exposed group, presenting normal caput tubules like in the control (F) BPB (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (G) BPB (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. Likewise, (H)BPF (5 µg/L) exposed group, presenting normal caput tubules like in the control (I) BPF (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (J) BPF (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. In the same way, (K) BPS (5 µg/L) exposed group, presenting normal caput tubules like in the control (L) BPS (25 µg/L) exposed group, presenting cauda tubules with sperm filled lumen (M) BPS (50 µg/L) exposed group presenting cauda tubules with less sperm in the lumen. H&E (40x).

DISCUSSION

Endocrine disrupting chemicals (EDCs) are synthetic or natural compounds which alter the endocrine functions often through mimicking or blocking the endogenous hormones (Schug *et al.*, 2011). Plasticizers and pesticides are often the main source of these synthetic EDCs. The actions of these EDCs on the endocrine system have resulted in the developmental deficits in many invertebrates and mammals (Crain *et al.*, 2007, Elango *et al.*, 2006, Kavlock *et al.*, 1996). Exposure in early life to these EDCs appear to have more sever effects and these endocrine disturbances persist through later life (Birnbaum and Fenton, 2003, Rubin, 2011, Rubin and Soto, 2009). In the present study we investigated possible effects of BPA and its analogues BPB, BPF and BPS different concentrations in drinking water in the prenatal development of male rats.

Endocrine disruptors research has shown that persistent exposure to low dose of these toxic and lethal chemicals lead into disturbed molecular, cellular and physiological functions (Maffini *et al.*, 2006, Vom Saal and Welshons, 2006, Vandenberg *et al.*, 2007). Present findings suggest that chronic exposure to low, moderate and high levels of BPA and its analogues had deleterious effects on the growth and sexual maturation and led to abnormal development. We have shown in this study that chronic exposure to low, moderate and high levels of BPA impacts the animals through post-natal growth and sexual maturation. Hormones from both pituitary and hypothalamus lead into the normal development of reproductive system and any abnormality in the levels of hormones can lead into abnormal development or poor reproductive efficiency. Gonadotropin releasing hormone (GnRH) in this regard has great role in the regulation of spermatogenesis and testosterone secretion in the testis (Amory and Bremner, 2003, Page *et al.*, 2008). For the normal onset of puberty GnRH plays an important role and it has great physiological function in the adult animals (Okamura *et al.*, 2013). GnRH is furthered controlled by another important member of hormone in the hypothalamus known as kisspeptin which regulates the normal pulsatile secretion of GnRH (Terasawa *et al.*, 2013). GnRH pulse also play important role the regulation of estrogen receptor which controls the onset of puberty and ovulation in mammals. Abnormal levels of estrogen due to EDCs may lead to abnormal development in rats (Terasawa *et al.*, 2013, Sukhbaatar *et al.*, 2013). Exposure to EDCs in rodents during the developmental stages has been observed to be associated with disturbed

reproductive functions at puberty (Bonefeld-Jørgensen *et al.*, 2001). EDCs exposure in human have been observed to induce alterations in the normal development of reproductive organs (Mouritsen *et al.*, 2010, Euling *et al.*, 2008). BPA and its analogues different concentrations were investigated and its effects were analyzed in the rats through water exposure routes (Rubin, 2011, Rochester and Bolden, 2015, Rosenfeldt and Linden, 2004, Chu *et al.*, 2005, Rubin *et al.*, 2001, Kang *et al.*, 2007, Ji *et al.*, 2013, Lee *et al.*, 2013, Naderi *et al.*, 2014, Ullah *et al.*, 2016). In the present study effects of different concentrations of BPA and its analogues BPB, BPF and BPS in the drinking water were checked by prenatal exposure in the offspring reproductive system.

It was observed previously that animals prenatally exposed to BPA induce weight gain in the offspring by inducing reproductive toxicity (Christiansen *et al.*, 2013, Larsson *et al.*, 2014, Eladak *et al.*, 2015). There have also been studies where it has been observed that BPA and some of its analogues have been found to be associated with obesity (Del Moral *et al.*, 2016, Altamirano *et al.*, 2015, Mandrup *et al.*, 2016).In the current study we observed increase in the body weight after the prenatal exposure to BPA, BPB, BPF and BPS in pups at PND 1 until adulthood. Organs weight did not increase and may be associated with low concentrations of BPA and its analogues BPB, BPF and BPS to which animals were exposed. Significant increase was observed in the groups exposed to high concentrations of BPA and its analogues BPB, BPF and BPS and in accordance with the previous studies (Rubin and Soto, 2009, Rubin *et al.*, 2001, Durando *et al.*, 2007, Cagen *et al.*, 1999a).

Development of reproductive organs and its physical examination has verified toxic effects of EDCs on the development of reproductive system in rodents and other animals. These examinations can highlight the state of development of many organs in the body. Such parameters have been used in the study as anogenital distance in animals (the distance between anus and genitals which reflect the state of development of reproductive system in rodents and mammals) (Kobayashi *et al.*, 2002, Thankamony *et al.*, 2009, Thankamony *et al.*, 2016, Liu *et al.*, 2014, Boudalia *et al.*, 2014).

In our current study no significant difference was observed in the anogenital distance of male rats exposed to different concentrations of BPA and its analogues as BPB, BPF and BPS as compared to control. Previously, there have similar studies where animals were exposed to different concentrations of BPA and some of its analogues in which they have not shown any

difference in the anogenital area (Ema *et al.*, 2001, Kobayashi *et al.*, 2012, Kobayashi *et al.*, 2002). Similarly, Nipple retention is also considered an important marker for the altered androgens at the time of development (Hyoung *et al.*, 2007, Christiansen *et al.*, 2013, Thankamony *et al.*, 2009). This can give an indication of an abnormal reproductive system at the time of puberty (Hotchkiss *et al.*, 2007). Parameters as such are now a days considered necessary for the detection of adverse effects of any EDCs exposure (McIntyre *et al.*, 2002, Hotchkiss *et al.*, 2007). There was no significant difference in the nipple retention of male rats. Similarly, these concentrations did not induce any change in the organ and body weight suggesting that the present exposure to low concentrations did not have adverse effects on the reproductive system of male rats.

Hormones play an important role in the initiation of puberty. In the present prenatal exposure study significant difference was observed in testosterone, progesterone, LH and FSH concentrations in all groups exposed to different concentrations of BPA and its analogues BPB, BPF and BPS and control. There have been several studies which have shown the same effect on the concentrations of different hormones after exposure to BPA or some of its analogues (Rosenmai *et al.*, 2014, Eladak *et al.*, 2015, Rochester and Bolden, 2015, Salian *et al.*, 2011, Rubin and Soto, 2009). Significant difference was observed in the hormones concentrations levels of these animals exposed to low and high concentrations of BPA and its analogues BPB, BPF and BPS and control. Which suggest that considerably low dose of BPA and its analogues also bring considerable effect on the development of many systems in the prenatal period (Rosenmai *et al.*, 2014, Rochester and Bolden, 2015, Rubin, 2011).

In the daily sperm production significant change was observed in the different groups of animals exposed to different concentrations of BPA and its analogues. Comparable difference was observed in the number of sperms in the caput/carpus of epididymis in the exposed groups which was also observed in the previous studies by (Salian *et al.*, 2011, Talsness *et al.*, 2009, Vom Saal *et al.*, 1998, Cagen *et al.*, 1999b). Other sperm parameters were also different in the treated groups discuss earlier by (Maffini *et al.*, 2006, Kubo *et al.*, 2001, Takai *et al.*, 2000).

Besides, the reduction in the LH and FSH levels we observed reduced testosterone concentrations, reduced DSP and number of sperm in epididymis exposed to different concentrations of BPA and its analogues BPB, BPF and BPS. Similarly, the reproductive organs

weights were also reduced in different concentrations exposed groups to BPA and its analogues BPB, BPF and BPS. Our results are in accordance with the different previous studies were BPA and its analogues have been observed to result in an increase in the adipogenesis and preadipocytes (Somm *et al.*, 2009, Héliès-Toussaint *et al.*, 2014, Ahmed and Atlas, 2016). BPA and its analogues have also been observed to be associated with obesity and high fat in the different organs in the body (Boucher *et al.*, 2016b, Del Moral *et al.*, 2016, Vom Saal *et al.*, 2012, Somm *et al.*, 2009). Our results are in relation with multiple studies with BPA and some of its analogues where LH and FSH reduced levels supported the histological alterations in the testis and reduction in sperm production (Brown, Schultz, Cloud, & Nagler, 2008; M. Chen et al., 2013; Eladak et al., 2015; Somm et al., 2009).

Histological results of testis revealed significant change in the morphology of testicular cells. This may be because of estrogen receptor in these organs which paly critical role in the spermatogenesis. Perversely, studies have shown that exposure to different concentration of BPA and its analogues BPB, BPF and BPS in the prenatal life increase in estrogens and reduces the testosterone concentrations (Kinch *et al.*, 2015, Moreman *et al.*, 2017, Rosenfeld, 2017, Sharpe, 2001, Akingbemi and Hardy, 2001, Eladak *et al.*, 2015, Ullah *et al.*, 2016, Ullah *et al.*, 2018) .

In the current study we observed that BPA and its analogues BPB, BPF and BPS at different low concentrations prenatally exposed to male rats have potential hazardous effects on spermatogenesis and lead into oxidative stress in the reproductive organs of male rats reducing the DSP and histopathological changes in the seminiferous tubule epithelium.

CONCLUSIONS

In conclusion, the results of the present study indicate the interactions between EDCs and reproductive system. However, BPA and its analogues BPB, BPF and BPS showed effects on sexual development recognizing that BPA and BPB, BPF and BPS exposure to mother during pregnancy may induce toxicity in the offspring. Low concentrations of BPA and its analogues can have effect on the organs and sexual development of adult rats. However, further studies are required to expose pregnant mothers to higher concentrations of BPA and its analogues BPB, BPF and BPS to determine the toxic effects in both male and female offspring.

ABSTRACT

Background: Bisphenol A (BPA) is one of the highly produced chemicals of the world mainly used for the production of commonly used materials like food packaging, dental sealants, thermal receipts and baby feeding bottles. Endocrine disrupting effects of BPA has shown its interactions with estrogen receptor both α/β and receptor of thyroid hormone. Due to high reproductive toxicity of BPA general public abandoned its use and manufactures introduced BPA analogues which are said to be safer than BPA. The rise in these BPA analogues are rising concerns and there are studies where these analogues have been found in consumer products, food and in human urine samples. The present study aims to investigate the reproductive effects of BPA and its analogues BPB, BPF and BPS on testicular development in rats exposed during the neonatal stage of life.

Methods: BPA, BPB, BPF and BPS were subcutaneously injected with different concentrations (5, 25 and 50 mg/kg in 50 ul castor oil) from postnatal day (PND) 1 to PND 10 in male rats. On PND 80 animals were dissected and different organs were collected for determining of endocrine alterations in the reproductive system.

Results: Hormonal analysis showed significant reduction in testosterone, Luteinizing hormone (LH) and follicle stimulation hormone (FSH) while, the levels of estradiol were observed to be high then control. Histopathological and morphometrical results of testicular tissues showed alterations in the different cells of testis and epididymis. There was also reduction observed in the number of sperm in the caput of epididymis and daily sperm production (DSP).

Conclusion: In the conclusion of the present study it was found out that neonatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS bring about alterations in the reproductive system of male rats by both altering the hormonal system as well as testicular internal cellular morphology.

INTRODUCTION

Bisphenol A (BPA) is one of the highly produced chemicals of the world mainly used for the production of commonly used materials like food packagings, dental sealants, thermal receipts and baby feeding bottles (Rochester, 2013). BPA main source of exposures are inhalation, ingestion and dermal contact (Kang *et al.*, 2006, Vandenberg *et al.*, 2007, Huang *et al.*, 2012). Studies of several agencies across the globe has shown measurable levels of BPA in the blood and urine samples of 90 % of general and occupational population (Vandenberg *et al.*, 2007, LaKind and Naiman, 2011, Geens *et al.*, 2011, Vom Saal *et al.*, 2007). Endocrine disrupting effects of BPA has shown its interactions with estrogen receptor both α/β and receptor of thyroid hormone (Dong *et al.*, 2011, Gould *et al.*, 1998, Kuiper *et al.*, 1998, Watson *et al.*, 2007). Studies have shown that BPA exerts impact on the male and female reproductive system (Peretz *et al.*, 2014, Vom Saal *et al.*, 2007). Epidemiological studies have indicated that BPA exposure is associated with impaired reproductive system in male and females and alterations in the hormone system (Dodge *et al.*, 2015, Goldstone *et al.*, 2015, Lassen *et al.*, 2014, Scinicariello and Buser, 2016, Barbonetti *et al.*, 2016). It has also been observed from many studies that BPA exposure affects sperm production, ovary functions, uterine morphology and hormones concentrations in the animal models (Siracusa *et al.*, 2018, Ferris *et al.*, 2016, Wang *et al.*, 2016). Due to high reproductive toxicity of BPA general public abandoned its use and manufactures introduced BPA analogues which are said to be safe than BPA (Geens *et al.*, 2009, Liao *et al.*, 2012b, Liao *et al.*, 2012a). These analogues are used in the infant feeding bottles, food packaging and sippy cups. Currently BPA analogues are used in many industries as crosslinking reagents in the plastic industries to produce BPA free materials. The rise in BPA analogues are rising concerns and there are studies where BPA analogues have been found in consumer products, food and in human urine samples (Shi *et al.*, 2013, Ye *et al.*, 2015, Liao *et al.*, 2012b). Studies have shown that these BPA analogues similar structure with BPA and it is expected that these analogues may have potential adverse effects on the male and female reproductive system (Eladak *et al.*, 2015, Bonefeld-Jørgensen *et al.*, 2007, Qiu *et al.*, 2015, Rosenmai *et al.*, 2014). There have been reports showing that these analogues interact with receptors for estrogens, aryl hydrocarbons and androgens (Liao *et al.*, 2012b, Kitamura *et al.*, 2005, Stossi *et al.*, 2014). Regarding BPA and its analogues very few studies are available showing its toxicity in the reproductive health of animals.

Bisphenol S (BPS) is an analogue of BPA used in a variety of common consumer products these days. BPA exposure occur through inhalation, dermal contact and digestion and currently it has been detected in indoor dust, food, personal care products and paper currency (Liao *et al.*, 2012b, Liao *et al.*, 2012c, Liao and Kannan, 2013, Siracusa *et al.*, 2018, Thoene *et al.*, 2018, Szczepańsk *et al.*, 2018). BPA has also been detected in the urine samples of human in several countries of the world and its concentrations were comparable with BPA (Wang *et al.*, 2015, Yu *et al.*, 2015, Xue *et al.*, 2015, Jin and Zhu, 2016, Liao and Kannan, 2014b). BPA has weak affinity for estrogen receptor (ER) and also acts as agonist to estrogen (Dreier *et al.*, 2015, Huang *et al.*, 2014). In a study it was also found that BPS has similar estrogenic and antiandrogenic activity as BPA (Rosenmai *et al.*, 2014). In *in vivo* studies it has been observed that BPS exerts toxicity in the reproductive system by increasing the uterine weight, inducing ROS in the testes, decreasing antioxidant enzymes and altering morphology of testicular tissues (Rosenmai *et al.*, 2014, Ullah *et al.*, 2016). BPS has also been observed to alter reproductive hormones as in a study it was observed that exposure to BPS increased plasma estradiol concentrations and decreased testosterone levels (Ji *et al.*, 2013, Naderi *et al.*, 2014, Chen *et al.*, 2016b). A study recently also showed that BPS exposure causes DNA damage and abnormal cytoskeleton structure of spermatogonia cell lines (Liang *et al.*, 2016).

Bisphenol F (BPF) is also a member of BPA family found in the dental coatings, food packaging and industrials floors (Rochester and Bolden, 2015). It has been detected in several daily use items such as dairy products, meat, seafood, cereals and fruits (Liao *et al.*, 2012b). BPF has also been found in many human tissues like liver and placenta (Liao and Kannan, 2013, Cabaton *et al.*, 2006). In a study it was found that in 60 % of US population was found with BPF measurable concentrations in their blood and urine samples and BPF also has similar potency for estrogen as BPA (Ye *et al.*, 2015, Stroheker *et al.*, 2003). Regarding reproductive outcomes of BPF limited data is available so far however, a study on female rats showed that short time exposure lead into increased uterine weight (Higashihara *et al.*, 2007). In male rats and human study it was observed that BPF exposure lead to altered testosterone secretions (Roelofs *et al.*, 2015, Eladak *et al.*, 2015).

Bisphenol B (BPB) is another analogue of BPA used mainly in the polymer industry for the manufacturing of phenolic resins. BPB has been found with endocrine disrupting potentials and also has strong anti-androgenic and estrogenic activities (Kitamura *et al.*, 2005, Yoshihara *et al.*,

2004, Cunha and Fernandes, 2010). Some studies have shown that BPB leak into the food and contaminate it. BPB has been detected in tomato samples, sera and in the women endometriosis with an alarming concentrations range (Cobellis *et al.*, 2009, Grumetto *et al.*, 2008). There are also reports of BPB where it has been detected in beverages, indoor dust and sea canned food (Cobellis *et al.*, 2009, Rosenmai *et al.*, 2014). BPB has also been found in the urine samples of human in conjugated form (Cunha and Fernandes, 2010, Rochester and Bolden, 2015). BPB also show strong similarities with the estrogenic nature of BPA (Kitamura *et al.*, 2005). Another study on BPB showed activated estrogenicity in the rat liver cells by causing DNA damage leading to high oxidative stress after BPB exposure (Rosenmai *et al.*, 2014). Due to the structural similarity of these analogues with BPA, they have also been found to act via endocrine disruption similar to BPA. Therefore, the question arise whether this shift towards the analogues is safer or more threatening to humans than BPA exposure?

Wide spread exposure of BPA has lead its association with many disorders like diabetes, obesity and reproductive diseases. Due to some of the restrictions on the use of BPA this condition led to a shift towards the use of BPA so called safe analogues. Consequently, these analogues have registered their presence felt in various environmental compartments as well as in food and beverages, further enhancing the risk of their general and occupational exposure. BPA and its analogues in adults rats has resulted in reduced epithelial cells and lots of other complacencies (Ullah *et al.*, 2018). Present study aims to investigate possible effects of BPA and its analogues BPB, BPF and BPS on testicular development in rats exposed during neonatal stage of life. Although the toxicity of BPA has been studied in detail but such information on its analogues is still scarce.

MATERIAL AND METHODS

Animals and treatments

A total of 65 male and 65 female Sprague Dawley male rats (150 ± 20 g were taken from Quaid-i-Azam University Animal Sciences Primate Facility, Islamabad, Pakistan) were used in this study. All the animals were kept in the facility with controlled conditions of a 12-h light and dark cycle, at 23 ± 2 °C, with relative humidity of 50 % ± 10 %, and all the animals had free access to food and water. All experimental procedures were carried out in full compliance with Quaid-i-Azam human care and laboratory Animals, approved by the Experimental Animals Ethical Committee of Quaid-i-Azam University, Islamabad, Pakistan. Adult female rats (n=65) were placed with male rats (n=65) in the breeding cages prior to the start of the experiment. Five females and five males rats were breed in large breeding cages. The day pups were born was considered as postnatal day 1 (PND1). Pups were counted and sex difference was determined by the anogenital distance (AGD) under a stereomicroscope. Male pups were counted, marked and were randomly recruited for the experiment. The male pups were randomly divided into thirteen different treatment groups by the method of randomized complete block design (Festing and Altman, 2002). The pups were administered with BPA, BPB, BPF and BPS (Sigma-Aldrich, St. Louis MO, USA) subcutaneously where BPA, BPB, BPF and BPS were incorporated at 0 (Control Caster Oil 50 µL), 5, 25 and 50 mg/kg BW/day in 50 µL castor oil. Each group was given the above mentioned dose for ten days subcutaneously according to the previous studies (Fernández *et al.*, 2010, Ahsan *et al.*, 2018a). Mothers of the treated pups were fed with pelleted food (Soy and Alfalfa free) and water was provided in PSU bottles ad libitum. On PND 23 after weaning period, treated male animals (n = 10 animals/ mother) were isolated from their mothers and kept in stainless steel cages and were fed with laboratory pelleted food (Soy and Alfalfa free) and water was available ad libitum in PSU bottles. Animals were kept in the cages for next three months and during this period different parameters were determined. The main reason for the selection of this experiment was to understand how endocrine disrupting chemicals induce different effects at the different stages of life. The adult animals exposed to chemicals have reversible effects later in life known as activational effects but the if animals are exposed to these chemicals at an early life this lead to irreversible changes known as organizational effects (Fernández *et al.*, 2010).

On Post-natal day (PND) 23 all the treated male pups were separated and kept in cages provided with standard feed and water for the next three months and different parameters were checked on the planned time throughout the examination period.

Body weight gain and determination of puberty onset

All the animals were weighed on the different planned days of the study and body weight gained was also obtained for the separate groups. For the determination of body weight gain and final body weight animals were weighed on PND 30, 45 and 80. All the animals were checked daily for any sign of toxicity and puerty was also checked through external signs described elsewhere by (Sachs and Meisel, 1979). Animals were checked daily from onwards of PND 35 for any sign of puberty and in different groups where puberty took place in animals was noted.

Sample collection

On PND 79 all animals were fasted overnight, and weighed on the day after the end of treatment PND 80. Blood samples were immediately collected and centrifuged at 3000 rpm for 10 min, and the plasma was stored at -20 °C until further biochemical analysis. Following euthanasia, different organs were removed surgically and weighed to calculate the organ/body weight ratio. Subsequently, reproductive organs (right testis and right epididymis) were also fixed in 10% formalin for histopathological examination with the remaining reproductive organs (left testis and left epididymis) stored at -80 °C for further biochemical and reproductive parameters analysis.

Gonadal somatic index (GSI) and relative weight of organs

GSI is an important parameter used for estimation of gonadal maturity in the animals. GSI was obtained for each animal according to the formula used by Barber and Blake (Barber and Blake, 2006).

Relative weight of the organs was determined according to the following formula $Relative$

$$\boldsymbol{GSI = \frac{Gonadal\ weight\ (g)}{Body\ organs\ weight\ (g)} \times 100}$$

For determination of relative weight under given formula was used

$$\boldsymbol{Relative\ weight}\ (\frac{\boldsymbol{mg}}{\boldsymbol{g}}) = \frac{\boldsymbol{Organ\ weight}\ (\boldsymbol{mg})}{\boldsymbol{Body\ weight}\ (\boldsymbol{g})}$$

Biochemical assays, hormonal analysis, histopathology, daily sperm production (DSP) and number of sperms in epididymis

Antioxidant enzymes

Antioxidant enzymes, hormonal assays, testicular tissues histology and DSP were done as explained in chapter 3. Number of sperm in the epididymis and sperm transient time was done as explained in chapter 4.

Statistical analysis

Dunnet,s multiple comparison tests which followed (ANOVA) was used for the comparison of different groups with control using Graph Pad Prism software. Values were expressed as Mean ± SEM and were considered significant at $P < 0.05$.

RESULTS

Effects of BPA and its analogues BPB, BPF and BPS on the initial and final body weight and body weight gain in adult male rats

Effects of BPA and its analogues BPB, BPF and BPS on the initial and final body weight and weight gain in the male rats from (PND) 1 to PND 10 are presented in table 33. There was difference observed in the final body weight of animals treated with different doses of BPA, BPB, BPF and BPS but that difference was not statistically different than control (Table 33).

Effect of BPA and its analogues BPB, BPF and BPS exposure on anu-genital distance (AGD) and nipple retention (NR) in adult male rats after neonatal exposure to different concentrations of bisphenols

AGD distance and NR in the different treatment groups and control is presented in table 34. Significant difference was not observed in the AGD in the male rats after post-natal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS. AGD in male rats from control group was 4.31 ± 0.97 mm, while in the highest concentrations exposed groups of BPA, BPB, BPF and BPS, AGD was 4.62 ± 1.11, 4.70 ± 0.19, 4.52 ± 1.12 and 4.71 ± 1.19 mm. There was little number of nipples seen in groups exposed to high (50 mg/kg) concentrations of BPA, BPB, BPF and BPS and control. On the other hand, there was no significant difference observed in the numbers in nipple in the prenatal stage of groups exposed to low concentrations (5 and 25 mg/kg) of BPA and its analogues BPB, BPF and BPS as compared to control. Average number of nipples in control (0.32 ± 0.03) was not statistically different as compared to the BPA, BPB, BPF and BPS exposed groups presented in table 33.

Table 33: Effect of neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS on body weight gain in adult rats

Treatments	Parameters				
	Initial body weight (g)	**Final body weight (g)**	**weight gain (g)**	**AGD in males (mm)**	**NR in males**
Control	5.36 ± 0.11	231.26 ± 3.68	225.90	4.31 ± 0.97	0.32 ± 0.08
BPA 5 mg/L	4.80 ± 0.37	242.33 ± 3.77	237.53	4.40 ± 0.91	0.37 ± 0.06
BPA 25 mg/L	4.86 ± 0.16	239.06 ± 3.43	234.20	4.36 ± 1.16	0.36 ± 0.07
BPA 50 mg/L	4.72 ± 0.23	238.31 ± 1.99	233.59	4.62 ± 1.11	0.38 ± 0.06
BPB 5 mg/L	5.40 ± 0.21	237.69 ± 3.07	232.29	4.52 ± 0.93	0.31 ± 0.07
BPB 25 mg/L	4.80 ± 0.25	237.55 ± 3.06	232.75	4.28 ± 1.19	0.36 ± 0.07
BPB 50 mg/L	4.62 ± 0.15	236.35 ± 2.45	231.73	4.70 ± 0.19	0.38 ± 0.08
BPF 5 mg/L	5.10 ± 0.17	238.35 ± 2.00	233.25	4.64 ± 0.87	0.36 ± 0.06
BPF 25 mg/L	4.96 ± 0.32	236.35 ± 2.45	231.39	4.21 ± 0.95	0.37 ± 0.07
BPF 50 mg/L	4.46 ± 0.27	236.33 ± 2.44	231.87	4.52 ± 1.12	0.32 ± 0.06
BPS 5 mg/L	5.04 ± 0.15	238.35 ± 2.00	233.31	4.38 ± 0.92	0.37 ± 0.07
BPS 25 mg/L	4.78 ± 0.24	237.17 ± 2.23	232.39	4.45 ± 1.14	0.32 ± 0.05
BPS 50 mg/L	4.86 ± 0.67	236.42 ± 4.01	231.56	4.71 ± 1.19	0.36 ±0.07

Effects of BPA and its analogues BPB, BPF and BPS on the organs weight in the adult male rats after neonatal exposure

All the male rats exposed to different concentrations of BPA and its analogues BPB, BPF and BPS were analyzed daily for preputial skin after PND 35. The day one of puberty was considered as the preputial skin separated in the pups. External signs of puberty analysis showed that BPA and its analogues BPB, BPF and BPS exposure did not have any effect on this parameter (Table 34).

Weight of the paired testis of adult male rats exposed to different concentrations of BPA and its analogues BPB, BPF and BPS from PND 1 to PND 10 is presented in table 34. Significant decrease in the paired testis weight was observed in BPA 50 mg/kg/day ($P < 0.05$) group when compared to the control. Similarly, BPB 50 mg/kg/day caused significant reduction ($P< 0.05$) in the weight of paired testis. On the other hand, BPF and BPS 50 mg/kg/day significantly decreased ($P < 0.05$) paired testis weight; however other doses of BPA, BPB, BPF and BPS did not decrease paired testis weight as compared to the control (Table 34).

There was significant difference observed in the GSI of adult rats exposed postnatally to different concentrations of BPA 50 mg/kg/day ($P< 0.05$) as compared to control. BPB 50 mg/kg/day treatment caused significant reduction ($P < 0.05$) when compared to control. Similarly, BPF 50 mg/kg/day was also observed with significant reduction in GSI after post-natal exposure to different concentrations of BPA, BPB, BPF and BPS as compared to control. BPS treatment also caused significant reduction ($P < 0.05$) at dose level of 50 mg/kg/day. There was no significant difference observed in 5 and 25 mg/kg groups of BPA and its analogues BPB, BPF and BPS when compared to the control group.

Table 34: Effect of neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS on organ weight in adult rats

Treatments	Parameters		
	Puberty onset (day)	**Paired testis (g)**	**GSI**
Control	44.22 ± 0.72	1.40 ± 0.02	0.96 ± 0.02
BPA 5 mg/L	44.93 ± 0.49	1.32 ± 0.01	0.91 ± 0.03
BPA 25 mg/L	44.17 ± 0.58	1.29 ± 0.03	0.90 ± 0.04
BPA 50 mg/L	43.40 ± 0.92	1.21 ± 0.04 *	0.82 ± 0.02*
BPB 5 mg/L	44.26 ± 0.60	1.34 ± 0.01	0.91 ± 0.01
BPB 25 mg/L	44.30 ± 0.61	1.27 ± 0.05	0.90 ± 0.03
BPB 50 mg/L	43.42 ± 0.40	1.22 ± 0.04 *	0.81 ± 0.02*
BPF 5 mg/L	43.55 ± 0.74	1.33 ± 0.02	0.91 ± 0.01
BPF 25 mg/L	44.09 ± 0.67	1.24 ± 0.06	0.90 ± 0.03
BPF 50 mg/L	43.22 ± 0.54	1.21 ± 0.05 *	0.82 ± 0.03*
BPS 5 mg/L	43.86 ± 0.52	1.32 ± 0.05	0.91 ± 0.02
BPS 25 mg/L	44.35 ± 0.69	1.25 ± 0.07	0.90 ± 0.03
BPS 50 mg/L	42.91 ±0.52	1.21 ± 0.04 *	0.82 ± 0.03*

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

Organs weights of the adult male rats after neonatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS

Different organs weight like paired testis weight, paired epididymis weight, seminal vesicle weight and prostate weight are presented in table 35. There was no significant difference observed in the relative and absolute epididymis weight of male postnatal rats exposed to different concentrations of BPA, BPB, BPF and BPS when compared with the control.

Absolute and relative seminal vesicle weights in different treatment groups showed significant difference in the male rats after neonatal exposure to different concentrations of BPA, BPB, BPF and BPS. Significant difference was observed in BPA 50 mg/kg/day ($P < 0.05$) when compared to the control. Similarly, BPB, BPF and BPS treatment caused significant reduction ($P < 0.05$) at dose level of 50 mg/kg/day, however, BPA, BPB, BPF and BPS 5 and 25 mg/kg/day did not have any effect on absolute and relative seminal vesicle weight as compared to the control group presented in table 35.

Absolute and relative seminal vesicle and prostate weights in different treatment groups of BPA, BPB, BPF and BPS showed significant difference presented in table 36. There was significant difference observed in BPA 50 mg/kg/day ($P < 0.05$) group when compared to the control group. Similarly, BPB, BPF and BPS treatment caused significant reduction ($P < 0.05$) at dose level of 50 mg/kg/day, however, there was no significant difference observed in the groups of BPA, BPB, BPF and BPS 5 and 25 mg/kg/day when compared to control group (Table 36). There was no significant difference observed in the other non-reproductive organs like adrenals, liver, kidney and retroperitoneal fat pad when compared to the control group in exposed neonatal male rats to different concentrations of BPA, BPB, BPF and BPS as presented in table 36.

Table 35: Effect of neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS on organ weight in adult rats

Treatments	Parameters			
	Absolute Paired Epididymis weight (g)	**Relative epididymis weight (g)**	**Absolute seminal vesical weight (g)**	**Relative seminal vesicle weight (g)**
Control	0.61 ± 0.01	2.52 ± 0.03	0.69 ± 0.01	2.17 ± 0.01
BPA 5 mg/L	0.59 ± 0.03	2.49 ± 0.05	0.65 ± 0.02	2.16 ± 0.02
BPA 25 mg/L	0.58 ± 0.03	2.46 ± 0.02	0.63 ± 0.02	2.14 ± 0.02
BPA 50 mg/L	0.57 ± 0.02	2.53 ± 0.03	0.60 ± 0.02 *	2.12 ± 0.03 *
BPB 5 mg/L	0.60 ± 0.01	2.48 ± 0.02	0.64 ± 0.01	2.16 ±0.04
BPB 25 mg/L	0.58 ± 0.02	2.57 ± 0.04	0.62 ± 0.03	2.16 ± 0.01
BPB 50 mg/L	0.57 ± 0.01	2.48 ± 0.02	0.60 ± 0.02 *	2.12 ± 0.02 *
BPF 5 mg/L	0.59 ± 0.02	2.48 ± 0.02	0.64 ± 0.03	2.15 ± 0.01
BPF 25 mg/L	0.58 ± 0.01	2.55 ± 0.04	0.62 ± 0.03	2.13 ± 0.03
BPF 50 mg/L	0.57 ± 0.02	2.50 ± 0.02	0.60 ± 0.01*	2.12 ± 0.01 *
BPS 5 mg/L	0.60 ± 0.01	2.43 ± 0.04	0.64 ± 0.03	2.16 ± 0.02
BPS 25 mg/L	0.58 ± 0.02	2.46 ± 0.03	0.63 ± 0.02	2.15 ± 0.01
BPS 50 mg/L	0.57 ± 0.01	2.46 ± 0.03	0.60 ± 0.01*	2.12 ± 0.02 *

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

Table 36: Effect of neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS on organ weight in adult rats

Treatments	Parameters					
	Absolute prostate weight (g)	Relative prostate weight (g)	Fat pad weight (g)	Kidney weight (g)	Liver weight (g)	Adrenals weight (mg)
Control	0.57±0.03	2.17±0.01	1.40±0.02	0.93±0.01	6.07±0.36	37.04±2.44
BPA 5 mg/L	0.45±0.04	2.16±0.02	1.41±0.01	0.91±0.01	5.06±0.39	37.08±2.36
BPA 25 mg/L	0.61±0.03	2.14±0.02	1.47±0.02	0.93±0.01	5.88±0.24	38.26±1.79
BPA 50 mg/L	0.44±0.01*	2.11±0.03*	1.50±0.02	0.90±0.02	6.13±0.27	38.73±2.20
BPB 5 mg/L	0.56±0.02	2.16±0.01	1.38±0.04	0.90±0.01	6.33±0.22	36.88±1.95
BPB 25 mg/L	0.53±0.01	2.16±0.01	1.47±0.03	0.92±0.02	6.22±0.17	38.17±2.16
BPB 50 mg/L	0.44±0.01*	2.12±0.03*	1.48±0.03	0.90±0.01	6.25±0.24	38.53±2.03
BPF 5 mg/L	0.60±0.05	2.15±0.02	1.38±0.02	0.91±0.01	5.89±0.20	36.68±2.23
BPF 25 mg/L	0.50±0.02	2.13±0.02	1.48±0.02	0.93±0.02	6.24±0.17	38.53±2.01
BPF 50 mg/L	0.45±0.02*	2.12±0.01*	1.48±0.02	0.90±0.01	6.48±0.18	38.53±2.05
BPS 5 mg/L	0.57±0.02	2.16±0.01	1.47±0.02	0.90±0.02	5.86±0.24	36.68±2.27
BPS 25 mg/L	0.50±0.01	2.15±0.02	1.49±0.03	0.92±0.02	6.21±0.19	37.86±2.20
BPS 50 mg/L	0.44±0.03*	2.12±0.03*	1.47±0.01	0.90±0.01	5.93±0.22	38.08±1.62

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

Antioxidant enzymes, LPO and ROS in the adult male rats after neonatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS

Antioxidant enzymes reduced to a significant level while ROS and LPO levels increased in the adult male rats testicular tissues after neonatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS presented in table 37. Activity of CAT was expressed as units/mg tissue and in BPA 50 mg/kg/day significant ($P < 0.05$) reduction was observed in the exposed group as compared to the control. Similarly, significant reduction was also observed in BPB 50 mg/kg/day ($P < 0.05$) group when compared to control. CAT activity also reduced in BPF 50 mg/kg/day as compared to the other group. In the BPS exposed groups there was only significant reduction observed in BPS 50 mg/kg/day group as compared to the control group. On the other hand, BPA, BPB, BPF and BPS 5 and 25 mg/kg/day groups did not show significant reduction in the cat activity when compared to the control presented in table 37.

Activity of SOD was expressed as (mU/ mg protein) as presented in table 37. Significant reduction was observed in BPA 50 mg/kg/day ($P < 0.01$) when compared to control. Similarly, BPB 50 mg/kg/day exposed group showed significant ($P < 0.01$) reduction in SOD activity as compared to the control. On the other hand, BPF 50 mg/kg/day significantly reduced ($P < 0.01$) SOD activity in the rat testicular tissues. BPS high dose group 50 mg/kg/day also ($P < 0.01$) reduced SOD activity. However, 5 mg/kg/day and 25 mg/kg/day exposed groups did not show significant reduction in the SOD activity after neonatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS (Table 37).

POD activity expressed as (U/mg protein) in the testis after neonatal exposure reduced significantly ($P < 0.05$) in BPA 50 mg/kg/day group as compared to control. Similarly, BPB 25 and 50 mg/kg/day also showed significant reduction ($P < 0.05$) in the activity of POD as compared to control. POD activity was reduced significantly ($P < 0.05$) in BPF 25 and 50 mg/kg/day treated group in comparison to the control group. Similarly, BPS treatment caused significant reduction ($P < 0.05$) at dose levels of 25 and 50 mg/kg/day when compared to the control group. However, there was no significant reduction observed in other treated groups of BPA, BPB, BPF and BPS when compared to control (Table 37).

LPO activity in the different treatment groups and control after chronic exposure is presented in table 37. There was significant increase ($P < 0.01$) observed in the BPA 50 µg/L group as compared to the control. All the high doses groups exposed to neonatal exposure of BPB, BPF and BPS (50 µg/L) showed significant increase ($P < 0.01$) in the LPO activity as compared to control. However, there was no significant difference observed in 5 µg/L and 25 µg/L groups of BPA and its analogues BPB, BPF and BPS as compared to the control as presented in table 37. ROS in the testicular tissues of adult male rats after neonatal exposed to different concentrations of BPA, BPB, BPF and BPS is presented in table 37. Significant increase was observed in BPA 50 mg/kg/day ($P < 0.01$) group when compared to the control. ROS activity increased significantly ($P < 0.01$) in BPB 50 mg/kg/day treated groups as compared to the control. Similarly, BPF treatment caused significant increase ($P < 0.01$) at 50 mg/kg/day dose level in comparison to the control. However, BPS 50 mg/kg/day significantly increased ($P <0.01$) ROS activity as compared to control. On the other hand, all the other doses (5 and 25 mg/kg/day) of BPA, BPB, BPF and BPS did not show significant reduction in the ROS activity as compared to the control (Table 37).

Plasma testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol concentrations in the animals after neonatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS

Plasma testosterone (ng/ml), LH (ng/ml), FSH (mIU/ml) and estradiol concentrations (pg/ml) are presented in table 38. Significant reduction was observed in BPA 50 mg/kg/day ($P < 0.05$) treated group when compared to the control. Testosterone concentrations reduced significantly ($P < 0.05$) in BPB 50 mg/kg/day treated group as compared to the control. Similarly, BPF caused significant reduction ($P < 0.05$) at dose level 50 mg/kg/day. On the other hand, BPS 50 mg/kg/day significantly reduced ($P < 0.05$) testosterone in the plasma, however other doses (5 and 25 mg/kg/day) of BPA and its analogues BPB, BPF and BPS did not reduce plasma testosterone as compared to the control group.

Mean ± SEM plasma estradiol concentrations in the male rats exposed to different doses of BPA and its analogues (5, 25 and 50 mg/kg/day) during neonatal period of life from PND 1 to PND 10 are presented in table 38.

Plasma estradiol concentrations in the animals exposed to BPA 50 mg/kg/day were significantly ($P < 0.001$) increased as compared to the control group. Estradiol concentration increased significantly ($P < 0.001$) in BPB 50 mg/kg/day treated group in comparison to the control group. Similarly, BPF treatment caused significant increased ($P <0.001$) at dose level of 50 mg/kg/day, however, BPF 5 and 25 mg/kg/day did not affect estradiol concentrations significantly as compared to the control. On the other hand, BPS 50 mg/kg/day group significantly increased ($P < 0.001$) estradiol concentrations; however, other groups (5 and 25 mg/kg/day) did not increase estradiol concentration as compared to the control.

Plasma LH concentrations in the male rats exposed to different doses of BPA and its analogues BPb, BPF and BPS (5, 25 and 50 mg/kg/day) during neonatal period of life from PND 1 to PND 10 were reduced in the treated groups as compared to the control (Table 38). Significant reduction was observed in BPA 50 mg/kg/day ($P < 0.001$) when compared to the control. Similarly, LH concentrations were reduced significantly ($P < 0.001$) in BPB 50 mg/kg/day treated groups in comparison to the control group. Similarly, BPF treatment caused significant reduction ($P < 0.001$) at dose level of 50 mg/kg/day as compared to control. BPS 50 mg/kg/day significantly reduced ($P < 0.001$) plasma LH concentrations in comparison to the control. However, other doses 5 and 25 mg/kg/day did not reduce plasma LH concentrations as compared to control.

Plasma FSH concentrations in the treatment groups were found reduced as compared to the control group as presented in table no 38. Significant reduction in plasma FSH levels ($P < 0.01$) was noted in the highest concentration (50 mg/kg/day) exposed group of BPA when compared to the control. FSH concentration was reduced significantly ($P < 0.01$) in BPB 50 mg/kg/day when compared to the control. Similarly, BPF treatment caused significant reduction ($P < 0.01$) at dose level of 50 mg/kg/day in comparison to the control. On the other hand, PBS 50 mg/kg/day significantly reduced ($P <0.01$) FSH concentration in plasma as compared to the control. However, other treatment groups (5 and 25 mg/kg/day) of BPA and its analogues BPB, BPF and BPS plasma FSH levels were reduced but were not statistically significant as compared to the control.

Table 37: Effect of neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS on the antioxidants profile in adult rats

Treatments	Parameters				
	CAT (U/mg protien)	**SOD (U/mg protien)**	**POD (U/mg protien)**	**LPO (U/mg protien)**	**ROS (U/mg protien)**
Control	7.28 ± 0.24	32.34 ± 0.29	6.25 ± 0.25	7.33 ± 0.31	094.70 ± 2.54
BPA 5 mg/L	6.11 ± 0.37	32.09 ± 0.68	5.55 ± 0.24	7.02 ± 0.15	095.15 ± 2.60
BPA 25 mg/L	6.03 ± 0.43	31.38 ± 0.43	5.60 ± 0.09	7.93 ± 0.19	100.57 ±5.27
BPA 50 mg/L	5.59 ± 0.41*	30.66 ± 0.33**	5.21 ± 0.26*	9.03 ± 0.23**	118.70 ± 4.83**
BPB 5 mg/L	6.51 ± 0.57	32.17 ± 0.30	5.45 ± 0.17	7.09 ± 0.39	096.35 ± 2.12
BPB 25 mg/L	5.79 ± 0.34	31.34 ± 0.31	5.31 ± 0.32*	7.38 ± 0.46	101.00 ± 5.77
BPB 50 mg/L	5.49 ± 0.39*	30.81 ± 0.20**	5.23 ± 0.23*	9.00 ± 0.33**	118.60 ± 3.52**
BPF 5 mg/L	6.54 ± 0.36	32.32 ± 0.24	5.45 ± 0.23	6.98 ±0.36	094.70 ± 2.40
BPF 25 mg/L	5.86 ± 0.28	31.14 ± 0.30	5.35 ± 0.23	7.34 ± 0.42	101.40 ± 4.24
BPF 50 mg/L	5.37 ± 0.33*	30.43 ± 0.11**	5.21 ± 0.10*	8.99 ± 0.22**	118.00 ± 5.05**
BPS 5 mg/L	6.28 ± 0.47	32.60 ± 0.17	5.43 ± 0.19	7.08 ± 0.35	094.84 ± 2.30
BPS 25 mg/L	5.86 ± 0.36	31.64 ± 0.16	5.26 ± 0.22*	7.36 ± 0.49	101.24 ± 2.80
BPS 50 mg/L	5.56 ± 0.45*	30.57 ± 0.15**	5.24 ± 0.20*	9.00 ± 0.25**	117.85 ± 7.06**

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

Table 38: Effect of neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS on plasma testosterone, estradiol, Luteinizing hormone and Follicle Stimulating hormone concentrations in adult rats

Treatments	Parameters			
	Testosterone (ng/ml)	**Estradiol (pg/ml)**	**LH (ng/ml)**	**FSH (mIU/ml)**
Control	4.86 ± 0.39	1.35 ± 1.01	1.71 ± 0.07	1.38 ± 0.22
BPA 5 mg/L	4.52 ± 0.37	1.33 ± 0.08	1.60 ± 0.04	0.89 ± 0.19
BPA 25 mg/L	4.45 ± 0.13	2.21 ± 0.36	1.49 ± 0.03	0.91 ± 0.16
BPA 50 mg/L	3.40 ± 0.33 *	3.68 ± 0.32 ***	1.20 ± 0.06 ***	0.55 ± 0.05 **
BPB 5 mg/L	4.50 ± 0.12	1.96 ± 0.40	1.58 ± 0.02	0.90 ± 0.21
BPB 25 mg/L	4.34 ± 0.32	2.10 ±0.48	1.53 ± 0.02	0.96 ± 0.21
BPB 50 mg/L	3.40 ± 0.33 *	3.63 ± 0.35 ***	1.23 ± 0.05 ***	0.52 ± 0.03 **
BPF 5 mg/L	4.33 ± 0.12	1.41 ± 0.05	1.57 ± 0.08	0.89 ± 0.16
BPF 25 mg/L	4.07 ± 0.21	2.46 ± 0.32	1.52 ± 0.05	0.92 ± 0.18
BPF 50 mg/L	3.34 ± 0.46 *	3.36 ± 0.34 ***	1.25 ± 0.06 ***	0.54 ± 0.02 **
BPS 5 mg/L	4.43 ± 0.58	1.51 ± 0.09	1.62 ± 0.06	0.89 ± 0.15
BPS 25 mg/L	3.76 ± 0.30	2.62 ± 0.48	1.51 ± 0.02	0.95 ± 0.24
BPS 50 mg/L	3.38 ± 0.46 *	3.87 ± 0.32 ***	1.26 ± 0.05 ***	0.52 ± 0.05 **

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

*****:** Indicate significance at $p < 0.01$ vs control

Effects of neonatal exposure of different concentrations of BPA and its analogues BPB, BPF and BPS on sperm motility, sperm viability and sperm count parameters

Sperm motility (%) in the control and different treated groups of BPA, BPB, BPF and BPS are presented in table 39. In the treated groups of BPA, BPB, BPF and BPS the percentage of sperm motility had decreased as compared to control. Significant reduction was observed in BPA 5 mg/kg/day ($P < 0.05$), BPA 25 mg/kg/day ($P < 0.01$) and BPA 50 mg/kg/kg ($P < 0.001$) when compared to the control. Sperm motility percentage was reduced significantly ($P < 0.01$ and $P < 0.001$) in BPB 25 and 50 mg/kg/day treated group as compared to control. Similarly, BPF treated groups caused significant reduction ($P < 0.01$ and $P < 0.001$) in the sperm motility as compared to control. On the other hand, BPF 25 and 50 mg/kg/day significantly reduced ($P < 0.01$ and $P < 0.001$) percentage of sperm motility as compared to the control. BPS treatment also caused significant reduction ($P < 0.01$ and $P < 0.001$) in the 25 and 50 mg/kg/day groups as compared to the control.

Sperm viability (%) in the control and different treated groups of BPA, BPB, BPF and BPS are presented in table 39. Significant reduction was observed in BPA 25 mg/kg/day ($P < 0.05$) and BPA 50 mg/kg/kg ($P < 0.001$) when compared to the control. Sperm viability % was reduced significantly ($P < 0.05$ and $P < 0.001$) in BPB 25 and 50 mg/kg/day treated group as compared to control. Similarly, BPF treated groups caused significant reduction ($P < 0.01$ and $P < 0.001$) in the sperm viability % as compared to control. BPS treatment also caused significant reduction ($P < 0.01$ and $P < 0.001$) in the 25 and 50 mg/kg/day groups as compared to the control. However, there was no significant difference observed in some of the low dose treated groups as compared to the control.

Mean ± SEM Daily sperm production (DSP) in the control and BPA and its analogues BPB, BPF and BPS treated groups are presented in table 39. In the treated group of BPA 50 mg/kg/day significant reduction ($P < 0.05$) in the DSP was observed when compared to control. Significant reduction was observed in BPB 50 mg/kg/day ($P < 0.05$) when compared to the control. DSP was also reduced significantly ($P < 0.05$) in BPF 50 mg/kg/day treated group as compared to control. On the other hand, BPS 50 mg/kg/day significantly reduced ($P < 0.05$) percentage of DSP as compared to the control. However, there was no significant difference observed in 5 and 25 mg/kg/day treated groups as compared to the control.

Sperm parameters in the treatment groups of BPA and its analogues BPB, BPF and BPS and control group showed variation in the number of sperm in the caput/carpus region of epididymis. In the caput/carpus epididymis sperm number showed significant reduction ($P < 0.001$) in BPA 50 mg/kg/day group when compared to control. Significant reduction ($P < 0.001$) in the DSP was noted in the BPB 50 mg/kg/day treated group than control. Significant reduction ($P < 0.001$) in BPB 50 mg/kg/day treated group as compared to control. On the other hand, BPF 50 mg/kg/day significantly reduced ($P < 0.001$) in the number of sperm in caput/carpus region as compared to the control. There was also significant reduction ($P < 0.001$) observed in BPS 50 mg/kg/day group as compared to control. However, there was no significant reduction observed in BPA 5 and 25 mg/kg/day groups when comparison was done with the control (Table 39).

Cauda epididymis sperm number in the treated groups of BPA and its analogues BPB, BPF and BPS and control showed alterations in the DSP. There was significant reduction ($P < 0.5$, $P < 0.001$ and $P < 0.001$) observed in the number of daily sperms in BPA 5, 25 and 50 mg/kg/day groups as compared to control. Similarly, significant reduction ($P < 0.01$, $P < 0.001$ and $P < 0.001$) was also observed in BPB, BPF and BPS 5, 25 and 50 mg/kg/day treated groups when compared to the control as presented in table 39.

Table 39: Daily sperm production (DSP), Sperm motility and sperm count parameters in the testis and different parts of epididymis in adult rats after neonatal exposure to different concentrations of Bisphenols as BPA, BPB, BPF and BPS

Treatments	Parameters				
	Sperm Motility %	**Sperm viability %**	**DSP x 10^6**	**Caput/ carpus epididymis sperm number ($x10^6$/g organ)**	**Cauda epididymis sperm number ($x10^6$/g organ)**
Control	77.82 ± 0.69	95.90 ± 0.51	73.37 ± 0.69	253.28 ± 0.79	402.96 ± 0.85
BPA 5 mg/L	74.88 ± 0.49*	94.44 ± 0.43	62.28 ± 0.37	249.73 ± 1.09	398.13 ± 0.58*
BPA 25 mg/L	72.73 ± 0.44***	93.46 ± 0.33*	62.51 ± 0.96	240.35 ± 3.11	394.95 ± 1.18***
BPA 50 mg/L	68.73 ± 0.90***	89.21 ± 0.53***	61.66 ± 0.72*	234.69 ± 1.73***	386.15 ± 1.80***
BPB 5 mg/L	75.24 ± 0.34	94.53 ± 0.73	62.37 ± 0.70	250.33 ± 0.70	397.35 ± 0.72**
BPB 25 mg/L	74.06 ± 0.95**	93.41 ± 0.46*	62.48 ± 1.81	244.82 ± 1.35	391.75 ± 0.52***
BPB 50 mg/L	69.55 ± 0.96***	88.34 ± 0.58***	61.50 ± 0.50*	238.35 ± 0.84***	388.33 ± 1.14***
BPF 5 mg/L	75.24 ± 0.33	94.84 ± 0.51	62.17 ± 5.85	250.13 ± 1.21	397.53 ± 0.58**
BPF 25 mg/L	73.99 ± 0.75**	93.19 ± 0.37**	60.62 ± 5.03	243.97 ± 1.01	394.53 ± 1.15***
BPF 50 mg/L	70.39 ± 0.74***	88.37 ± 0.59***	61.43 ± 0.41*	237.93 ± 0.77***	388.55 ± 0.74***
BPS 5 mg/L	75.06 ± 0.35	95.04 ± 0.72	60.28 ± 3.52	250.71 ± 0.67	397.53 ± 0.48**
BPS 25 mg/L	74.31 ± 0.80**	93.21 ± 0.35**	62.33 ± 5.25	243.60 ± 0.94	392.35 ± 0.82***
BPS 50 mg/L	69.99 ± 0.83***	87.97 ± 0.52***	61.56 ± 0.69*	238.55 ± 0.38***	386.13 ± 1.71***

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

*****:** Indicate significance at $p < 0.01$ vs control

Effects of neonatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS on histological and planimetry changes of testicular tissues in adult male rats

Histological study of the microscopic slides of the testicular tissues revealed normal morphology of the structures in the control and 5 μg/L exposed groups. The seminiferous tubules were compactly arranged with sperm filled lumen and the interstitial space was relatively thin in the groups treated with BPB and its analogues BPB, BPF and BPS. In the groups exposed to 25 μg/L and 50 μg/L of BPA and its analogues BPB, BPF and BPS the tubules were relatively small with larger interstitial spaces and less filled lumen. Cellular arrest at spermatogoneal stage and at round spermatids were more evident in the highest concentration (50 μg/L) exposed groups as compared to the control. In 25 μg/L exposed group, cellular arrest was observed but was less than 50 μg/L exposed group shown in fig 17.

Planimetry results showed significant ($P < 0.01$ and $P < 0.001$) reduction in the percentage area of seminiferous tubules in the group, exposed to 25 and 50 mg/kg/day of BPA as compared to control. Significant reduction was observed in BPB 5, 25 and 50 mg/kg/day ($P < 0.05$, $P < 0.01$ and $P < 0.001$) groups when compared to the control. Similarly, BPF treatment caused significant reduction ($P < 0.01$, $P < 0.001$ and $P < 0.001$) at dose level of 5, 25 and 50 mg/kg/day groups as compared to control. On the other hand, BPS 5, 25 and 50 mg/kg/day significantly reduced ($P < 0.01$, $P < 0.01$ and $P < 0.001$) in the area of seminiferous tubules when compared to the control as presented in table 40.

Percentage area of interstitial space was reduced significantly ($P < 0.05$) in BPA 50 mg/kg/day group as compared to control. Significant reduction was observed in BPB 50 mg/kg/day ($P < 0.05$) group when compared to the control. Similarly, BPF treatment caused significant reduction ($P < 0.01$) at dose level of 50 mg/kg/day group in comparison to the control group. On the other hand, BPS 50 mg/kg/day significantly reduced ($P < 0.05$) in the area of area of interstitial space when compared to the control. However, there was no significant difference observed in BPA and its analogues BPB, BPF and BPS 25 and 50 mg/kg/day groups when compared to the control as presented in table 8.

In the percentage area of lumen there was significant reduction ($P < 0.05$) observed in BPA 50 mg/kg/day group when compared to control group as presented in table 40. Significant reduction

($P < 0.05$) was observed in BPB 50 mg/kg/day when compared to the control. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose level of 50 mg/kg/day group as compared to control. On the other hand, BPS 50 mg/kg/day significantly reduced ($P < 0.05$) in the area of lumen tubules when compared to the control (Table 40). However, there was no significant difference observed in BPA and its analogues BPB, BPF and BPS 25 and 50 mg/kg/day groups when compared to the control (Table 40).

Area of epithelium % is presented in table 8. There was no significant difference observed in all the neonatal BPB and its analogues BPB, BPF and BPS treatment groups when compared to the control.

In the seminiferous tubules diameter there was significant reduction ($P < 0.05$) observed in BPA 50 mg/kg/day group when compared to control. Significant reduction ($P < 0.05$) was observed in BPB 50 mg/kg/day when compared to the control. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose level of 50 mg/kg/day group as compared to control. On the other hand, BPS 50 mg/kg/day significantly reduced ($P < 0.05$) in the seminiferous tubules diameter when compared to the control presented in table 40. However, there was no significant difference observed in BPA, BPB, BPF and BPS 25 and 50 mg/kg/day groups when compared to the control.

Seminiferous tubules epithelial height is presented in table 40. There was significant difference ($P < 0.05$) observed in BPA 50 mg/kg/day neonatal exposed group as compared to the control group. Significant reduction ($P < 0.05$) was observed in BPB 50 mg/kg/day when compared to the control. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose level of 50 mg/kg/day group as compared to control. On the other hand, BPS 50 mg/kg/day significantly reduced ($P < 0.05$) in the seminiferous tubules diameter when compared to the control. However, there was no significant difference observed in BPA, BPB, BPF and BPS 25 and 50 mg/kg/day groups when compared to the control.

Table 40. Histopathological study of testicular tissues of adult rats after neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS

Treatments	Parameters					
	Area % of seminiferous tubule	Area % of interstitial space	Area % of Lumen	Area % of epithelium	Seminiferous tubules diameter (µm)	Seminiferous Tubule epithelial height (µm)
Control	95.53 ± 1.33	13.57 ± 0.48	17.25 ± 0.62	85.36 ± 0.83	232.32 ± 0.53	68.23 ± 0.99
BPA 5 mg/L	89.48 ± 0.55	15.41 ± 0.32	16.01 ± 0.34	85.30 ± 0.81	228.22 ± 1.81	63.73 ± 2.36
BPA 25 mg/L	88.91 ± 1.69*	16.33±0.54*	15.75 ± 0.37	85.50 ± 0.83	225.97 ± 1.90	60.28±2.37*
BPA 50 mg/L	86.06 ± 0.43***	17.50 ± 1.34	15.42±0.40*	85.55 ± 0.75	223.95±1.95*	59.80±0.90*
BPB 5 mg/L	88.21 ± 2.56*	15.91 ± 0.82	16.16 ± 0.41	85.32 ± 0.80	228.64 ± 1.98	63.68 ± 2.19
BPB 25 mg/L	87.28 ± 0.71**	16.40 ± 0.66	15.88 ± 0.36	85.45 ± 0.77	225.75 ± 1.90	62.33 ± 2.36
BPB 50 mg/L	86.31 ± 0.91***	17.99±1.03**	15.44±0.39*	85.57 ± 0.85	224.92±1.66*	61.06±0.36*
BPF 5 mg/L	87.61 ± 0.64**	15.57 ± 0.51	15.98 ± 0.35	85.37 ± 0.82	227.42 ± 1.31	61.90 ± 2.30
BPF 25 mg/L	86.82 ± 0.95**	16.77 ± 0.63	15.85 ± 0.39	85.55 ± 0.82	226.37 ± 2.03	60.74 ± 2.26
BPF 50 mg/L	85.91 ± 2.90 ***	17.20±1.11*	15.42±0.35*	85.55 ± 0.75	224.72±0.92*	59.86±1.09*
BPS 5 mg/L	87.37 ± 0.95**	15.37 ± 0.67	16.08 ± 0.47	85.35 ± 0.83	228.24 ± 1.82	62.48 ± 2.23
BPS 25 mg/L	87.74 ± 0.52**	16.55 ± 0.76	15.78 ± 0.36	85.52 ± 0.83	226.17 ± 1.84	61.30 ± 2.17
BPS 50 mg/L	85.86 ± 2.39***	17.76±1.40*	15.44±0.39*	85.53 ± 0.76	224.32±1.81*	60.01±1.16*

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

*****:** Indicate significance at $p < 0.01$ vs control

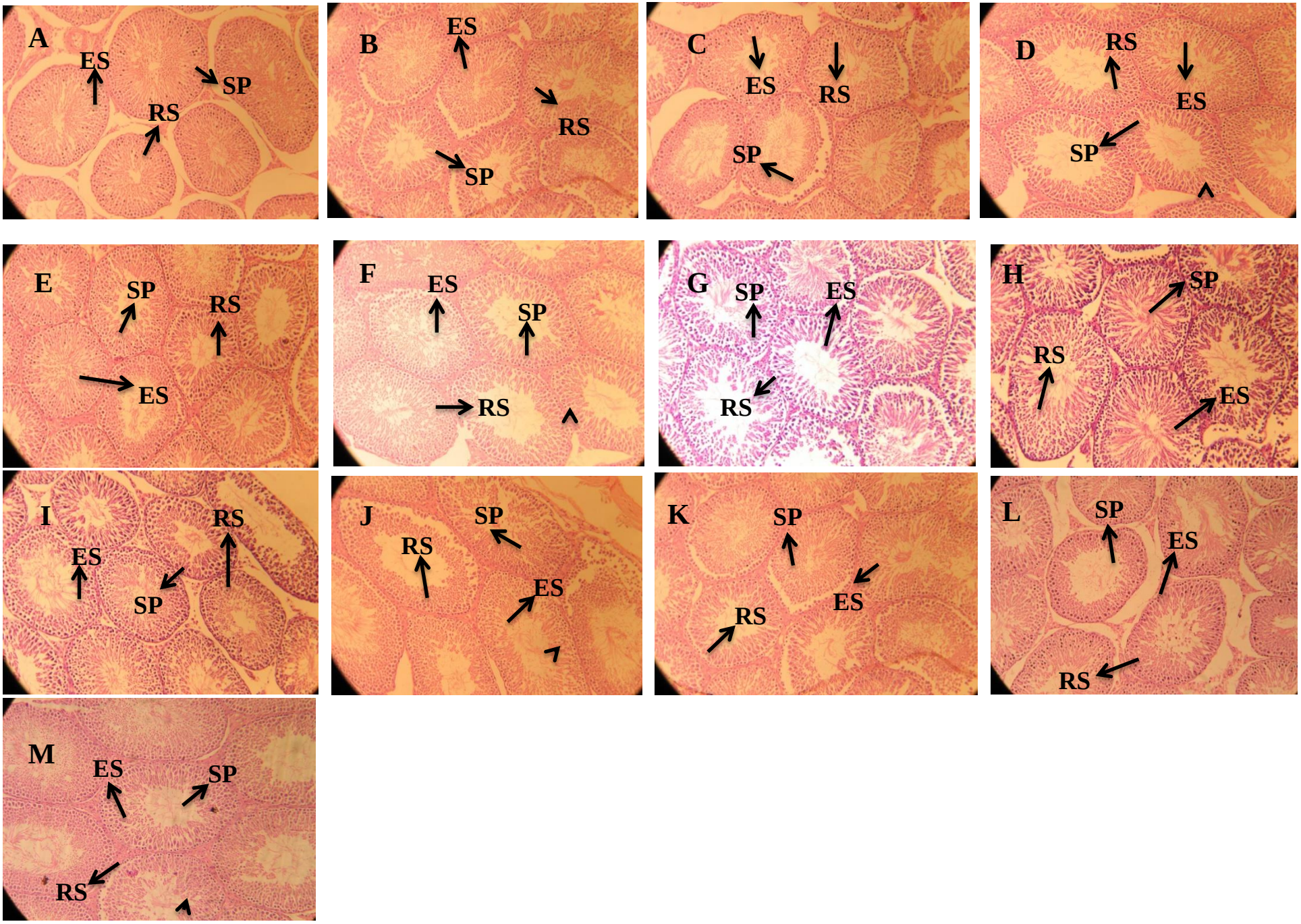
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Fig 17: Photomicrograph from testicular tissue showing (A) control; having thick epithelium with normal spermatogonia (SP), Round spermatids (RS), Elongated spermatids (ES) and filled lumen with sperm (B, C and D); BPA (5, 25 and 50 mg/kg/day) treatment presenting seminiferous tubules with epithelium (Line without arrow head) and spermatids (White arrow); (E, F and G) BPB (5, 25 and 50 mg/kg/day) treatment presenting seminiferous tubules with epithelium (Line without arrow head) and elongating spermatids (White arrow); (H, I and J) BPF (5, 25 and 50 mg/kg/day) treatment presenting seminiferous tubules with epithelium (Line without arrow head) and elongating spermatids (White arrow); (K, L and M) BPS (5, 25 and 50 mg/kg/day) treatment presenting seminiferous tubules with epithelium (Line without arrow head) and spermatids (White arrow). H&E (40X).

Effects of neonatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS on the number of different cells types in seminiferous tubules in the testis of adult rats

Number of different cells in the seminiferous tubules of male rat testis is presented in table 41. Significant reduction in the number of spermatogonia was observed in the group exposed to BPA 50 mg/kg/day ($P < 0.05$) than control group. Significant reduction was also observed in BPB 50 mg/kg/day ($P < 0.05$) when compared to the control. Similarly, BPF treatment caused significant reduction ($P < 0.01$) at dose level of 50 mg/kg/day as compared to control. On the other hand, BPS 50 mg/kg/day significantly reduced ($P < 0.01$) number of spermatogonia as compared to control. However, BPA and its analogues BPB, BPF and BPS 5 and 25 mg/kg/day did not significantly reduce number of spermatogonia as compared to control.

In the number of spermatocytes significant reduction was observed in BPA 50 mg/kg/day ($P < 0.01$) when compared to the control. Spermatocytes number was reduced significantly ($P < 0.01$) in BPB 50 mg/kg/day treated group as compared to control group. Similarly, BPF 50 mg/kg/day treatment caused significant reduction ($P < 0.01$) at dose level of 50 mg/kg/day as compared to control. BPS 50 mg/kg/day treated group significantly reduced ($P < 0.01$) the number of spermatocytes when compared to the control. On the other hand, the other doses of BPA and its analogues BPB, BPF and BPS did not reduce number of spermatocytes as compared to the control (Table 41).

Number of spermatids in different treatment groups and control is presented in table 41. Significant reduction was observed in BPA 50 mg/kg/day ($P < 0.05$) when compared to the control. Spermatids number reduced significantly ($P < 0.05$) in BPB 50 mg/kg/day treated group. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose level of 50 mg/kg/day as compared to control group. BPS 50 mg/kg/day treated group was also observed with significantly reduced ($P < 0.05$) number of spermatids as compared to the control. However, there was no significant difference observed in BPA and its analogues BPB, BPF and BPS 5 and 25 mg/kg/day groups when compared to the control (table 41).

Table 41. Seminiferous tubules number and different types of cell in the testis of adult rats after neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS

Treatments	Parameters		
	Spermatogonia (n)	**Spermatocytes (n)**	**Spermatids (n)**
Control	65.00 ± 1.17	76.44 ± 0.98	257.78 ± 2.07
BPA 5 mg/L	62.22 ± 1.40	73.11 ± 2.22	247.50 ± 4.70
BPA 25 mg/L	61.39 ± 1.43	72.44 ± 1.42	246.94 ± 4.34
BPA 50 mg/L	58.89 ± 1.04*	67.22 ± 1.39 **	241.00 ± 3.12 *
BPB 5 mg/L	62.33 ± 1.02	72.67 ± 1.79	247.72 ± 2.75
BPB 25 mg/L	60.44 ± 1.01	76.06 ± 2.19	246.06 ± 3.62
BPB 50 mg/L	59.00 ± 0.94 *	67.94 ± 1.02 **	242.56 ± 4.48 *
BPF 5 mg/L	62.11 ± 1.14	72.94 ± 2.20	246.83 ± 3.35
BPF 25 mg/L	60.50 ± 1.38	70.44 ± 1.79	245.11 ± 3.60
BPF 50 mg/L	58.00 ± 1.33 **	67.72 ± 1.48 **	240.06 ± 2.84 *
BPS 5 mg/L	63.06 ± 1.36	72.11 ± 2.10	246.22 ± 3.99
BPS 25 mg/L	62.67 ± 1.73	71.39 ± 1.75	245.94 ± 4.59
BPS 50 mg/L	58.83 ± 1.17 **	67.22 ± 1.39 **	240.61 ± 2.78 *

Values are presented as Mean ± SEM

*: Indicate significance at $p < 0.05$ vs control

**: Indicate significance at $p < 0.01$ vs control

Effects of neonatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS on the planimetry and morphological changes in the caput region of epididymis of adult rats

Caput epididymis and its different cells area, diameter and height is presented in table no 10. Male neonatal rats exposed to BPA and its analogues BPB, BPF and BPS expressed no significant changes in the histological results of caput epididymis.

Epididymis caput region planimetry results did not show significant reduction in the tubular diameter in the groups exposed to different concentrations of BPA, BPB, BPF and BPS as compared to the control. There was also no significant difference observed in the other parameters as lumen diameter, epithelial height and area covered with epithelium and lumen of different treatment groups when compared to the control (Table 42 and fig 18). There was very slight difference observed in the morphological difference of caput region of epididymis among the different treatment groups and control. In the different treatment groups 50 mg/kg/day of BPA and its analogues BPB, BPF and BPS slightly reduced number of sperm in the lumen when compared to the control. There was no significant difference observed in the other exposed groups in comparison to the control as shown in fig 18.

Effects of neonatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS on the planimetry and morphological changes in the cauda region of epididymis of adult rats

Planimetry and morphological results of the cauda region of the epididymis showed no significant alterations in the tubular diameter of the groups exposed to different concentrations of BPA, BPB, BPF and BPS. Mean ± SEM diameter of cauda tubules and lumen, epithelial height and area covered by epithelium and lumen in cauda epididymis are presented in table 43. Similarly, other parameters like lumen diameter did not show any significant alterations compared to the control. Morphological difference observed in the cauda region of epididymis showed only slightly reduced number of sperm in the lumen in 50 mg/kg/day exposed groups with different concentrations of BPA and its analogues BPB, BPF and BPS for 48 weeks of chronic exposure. No significant alterations were obvious in other groups in comparison with control (Table 43 and fig 19).

Table 42. Results of the caput epididymis morphometry in the adult rats after neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS

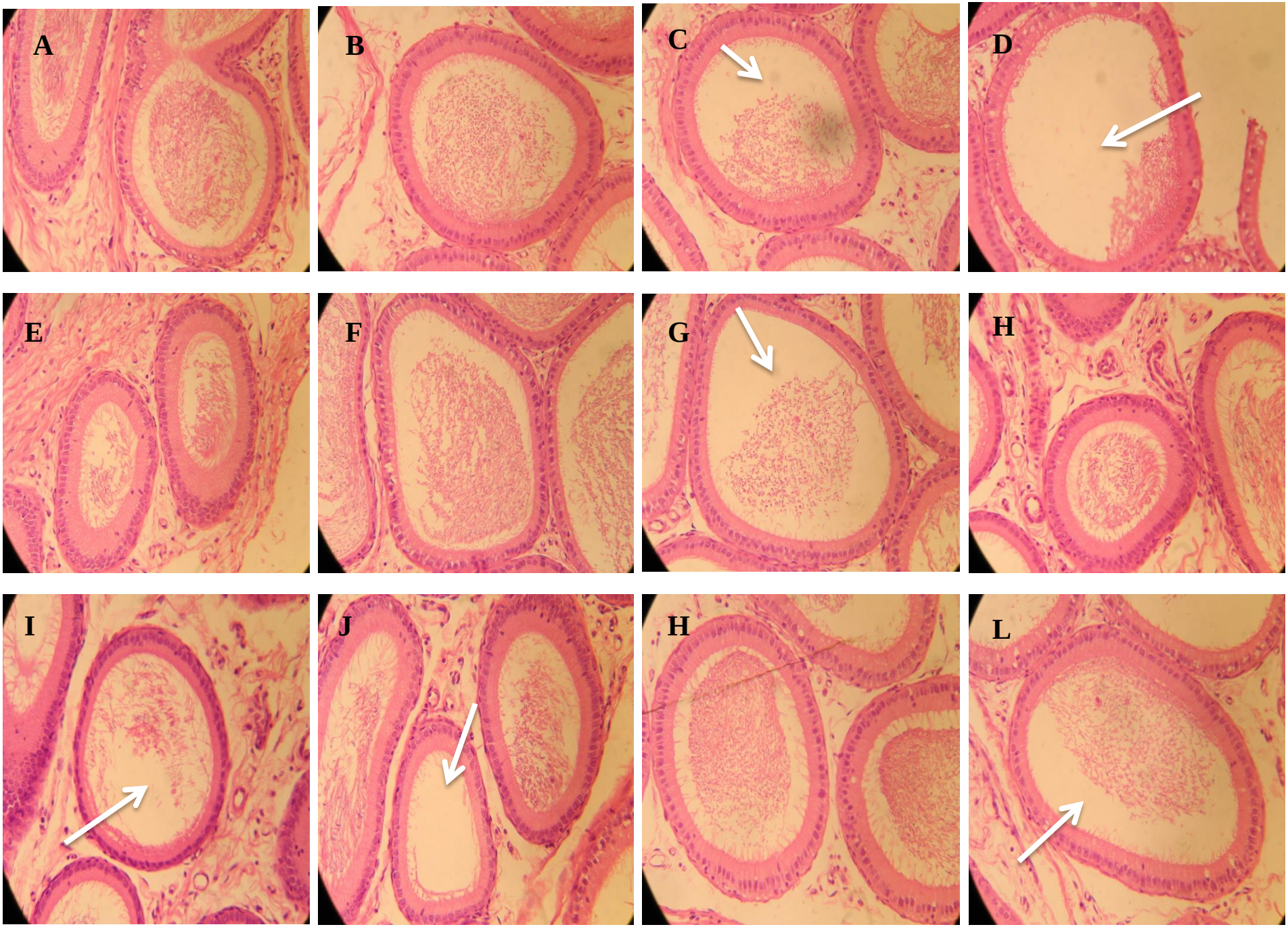
Treatments	Parameters				
	Tubular diameter (µm)	Lumen diameter (µm)	Epithelial height (µm)	Epithelium (%)	Lumen (%)
Control	340.40 ± 2.24	248.00 ± 20.34	28.00 ± 3.01	35.25 ± 3.67	72.75 ± 5.08
BPA 5 mg/L	338.80 ± 3.45	242.60 ± 1.16	27.40 ± 2.29	34.00 ± 2.60	71.70 ± 2.32
BPA 25 mg/L	336.20 ± 3.08	240.00 ± 0.83	26.00 ±1.89	33.51 ± 1.99	70.50 ± 1.83
BPA 50 mg/L	333.20 ± 3.99	239.20 ± 1.95	25.80 ± 2.22	31.25 ± 2.93	66.25 ± 2.85
BPB 5 mg/L	337.00 ± 4.93	242.60 ± 3.37	27.20 ± 1.71	34.94 ± 1.49	71.50 ± 4.86
BPB 25 mg/L	336.40 ± 4.22	240.20 ± 2.24	27.60 ± 3.04	33.65 ± 2.08	70.75 ± 5.25
BPB 50 mg/L	331.20 ± 1.35	237.80 ± 3.15	24.75 ± 2.14	31.16 ± 2.38	67.70 ± 3.49
BPF 5 mg/L	338.40 ± 4.06	242.80 ± 2.26	27.60 ± 2.52	34.65 ± 1.63	69.90 ± 2.07
BPF 25 mg/L	335.20 ± 1.39	241.80 ± 2.88	26.80 ± 1.77	33.05 ± 2.47	67.25 ± 2.17
BPF 50 mg/L	332.80 ± 3.08	239.20 ± 1.10	25.80 ± 2.03	31.20 ± 2.42	66.30 ± 1.68
BPS 5 mg/L	337.60 ± 3.10	242.60 ± 2.73	27.60 ± 1.46	35.40 ± 2.53	70.90 ± 2.03
BPS 25 mg/L	335.80 ± 1.98	241.80 ± 2.47	26.00 ± 2.68	32.50 ± 3.25	68.05 ± 2.11
BPS 50 mg/L	333.40 ± 1.32	239.80 ± 2.13	25.40 ± 1.83	30.00 ± 2.40	67.70 ± 0.95

Values are presented as Mean ± SEM

Table 43. Results of the cauda epididymis morphometry in the adult rats after neonatal exposure to different concentrations of bisphenol A and it's so called safe analogues BPB, BPF and BPS

Treatments	Parameters				
	Tubular diameter (um)	Lumen diameter (um)	Epithelial height (um)	Epithelium (%)	Lumen (%)
Control	444.40 ± 2.76	416.80 ± 3.73	30.65 ± 1.65	34.85 ± 3.81	68.95 ± 2.45
BPA 5 mg/L	441.60 ± 0.67	413.80 ± 2.10	29.50 ± 2.72	33.10 ± 2.07	69.30 ± 1.62
BPA 25 mg/L	441.40 ± 3.26	412.20 ± 4.30	28.70 ± 2.07	30.50 ± 2.53	68.50 ± 1.92
BPA 50 mg/L	441.00 ± 2.12	411.20 ± 3.45	28.20 ± 2.22	28.95 ± 1.18	71.25 ± 1.16
BPB 5 mg/L	440.60 ± 1.43	414.60 ± 2.24	29.60 ± 1.24	30.70 ± 7.52	69.50 ± 1.66
BPB 25 mg/L	441.60 ± 2.37	416.80 ± 2.22	28.00 ± 1.70	28.45 ± 3.02	69.95 ± 2.01
BPB 50 mg/L	441.00 ± 3.16	415.80 ± 2.05	27.60 ± 1.88	27.95 ± 1.56	71.65 ± 1.44
BPF 5 mg/L	440.80 ± 2.26	415.60 ± 2.50	29.80 ± 2.70	32.70 ± 1.55	69.90 ± 1.66
BPF 25 mg/L	440.60 ± 2.54	414.40 ± 2.33	28.80 ± 3.16	31.55 ± 3.21	71.45 ± 1.84
BPF 50 mg/L	451.00 ± 4.04	414.20 ± 2.43	28.20 ± 1.95	29.10 ± 5.89	71.70 ± 3.47
BPS 5 mg/L	441.40 ± 2.87	415.20 ± 4.66	29.00 ± 2.62	31.10 ± 3.40	69.70 ± 2.05
BPS 25 mg/L	441.00 ± 0.94	415.00 ± 2.12	28.80 ± 3.16	29.35 ± 1.86	70.05 ± 0.73
BPS 50 mg/L	441.80 ± 0.86	414.60 ± 2.24	27.60 ± 1.46	29.10 ± 2.95	71.50 ± 3.60

Values are presented as Mean ± SEM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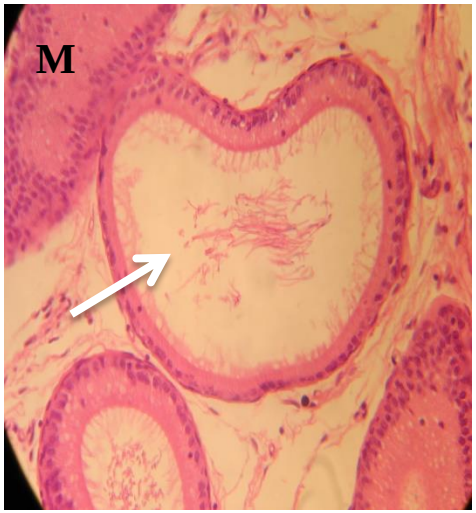
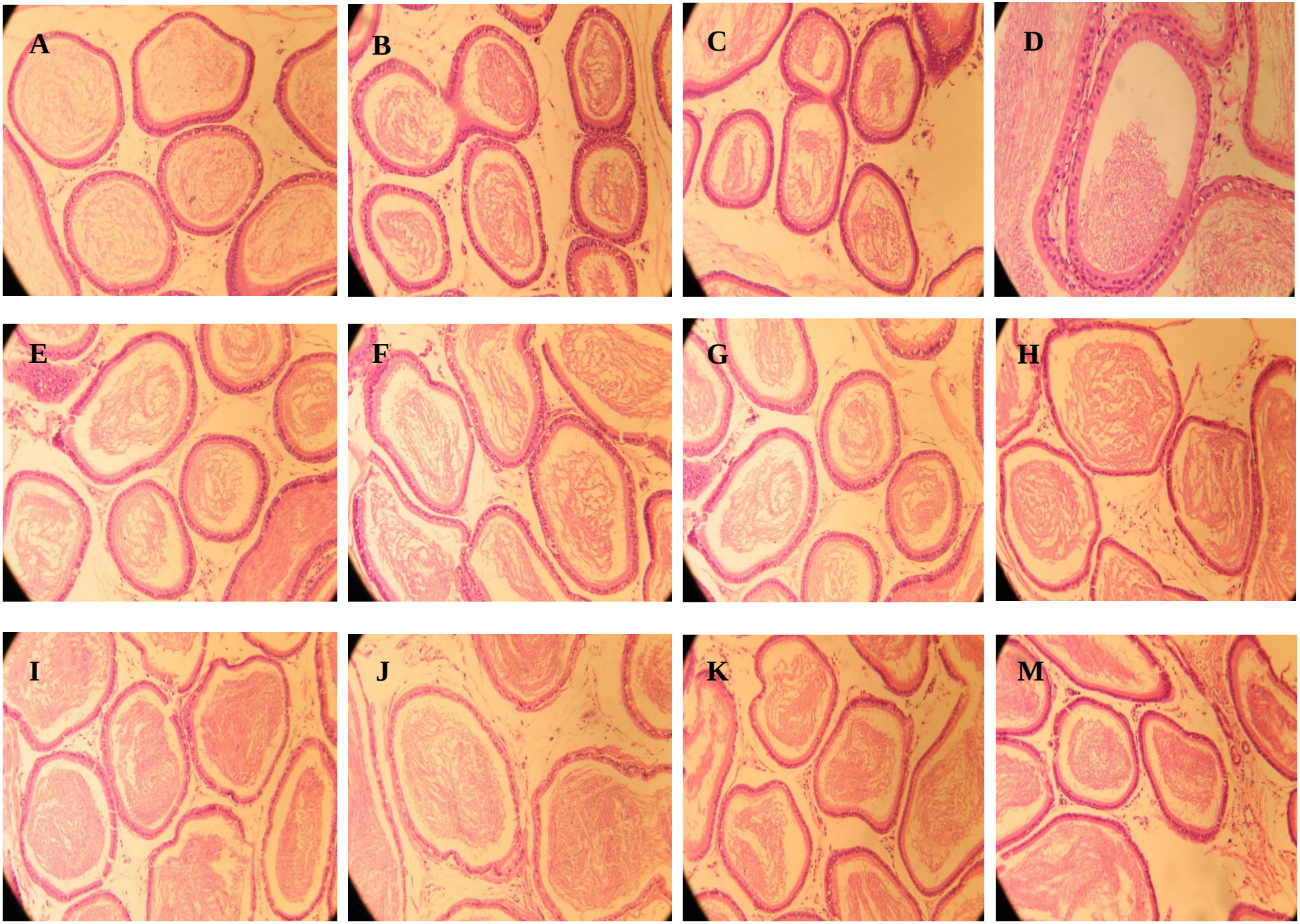


Fig 18: Photomicrograph of caput epididymis tissue showing (A) control; with compact arrangement of caput tubules with sperm filled lumen (B) BPA (5 mg/kg/day) exposed group, presenting normal caput tubules like in the control (C), BPA (25 mg/kg/day) exposed group showing seminiferous tubules with less number of sperm in the lumen (Arrow) and (D) BPA (50 mg/kg/day) exposed group presenting caput tubules with empty lumen (Arrow). Similarly, (E) BPB (5 mg/kg/day) exposed group, presenting normal caput tubules, (F) BPB (25 mg/kg/day) exposed group showing less number of sperms in the lumen, (G) BPB (50 mg/kg/day) exposed group showing less number of sperms and empty lumen (Arrow). (H) BPF (5 mg/kg/day) exposed group, presenting normal caput tubules, (I) (25 mg/kg/day) exposed group showing seminiferous tubules with less number of sperm in the lumen (Arrow) and (J) BPF (50 mg/kg/day) exposed group showing less number of sperms and empty lumen (Arrow). K and L BPS (5 and 25 mg/kg/day) exposed groups showing caput tubules with less number of sperms in the lumen and (M) BPS (50 mg/kg/day) exposed group presenting less number of sperms and empty lumen. H&E (40X).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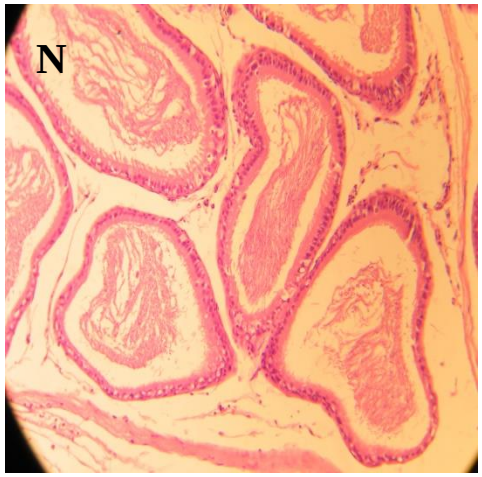

Fig 19: Photomicrograph of cauda epididymis tissue showing (A) control; with compact arrangement of cauda tubules with sperm filled lumen, (B) BPA (5 mg/kg/day) exposed group, presenting normal caput tubules like in the control, (C) BPA (25 mg/kg/day) exposed group, presenting cauda tubules with sperm filled lumen, (D) BPA (50 mg/kg/day) exposed group presenting cauda tubules with less sperm in the lumen. Similarly, (E) BPB (5 mg/kg/day) exposed group, presenting normal caput tubules like in the control, (F) BPB (25 mg/kg/day) exposed group, presenting cauda tubules with sperm filled lumen, (G) BPB (50 mg/kg/day) exposed group presenting cauda tubules with less sperm in the lumen. Likewise, (H) BPF (5 mg/kg/day) exposed group, presenting normal caput tubules like in the control, (I) BPF (25 mg/kg/day) exposed group, presenting cauda tubules with sperm filled lumen, (J) BPF (50 mg/kg/day) exposed group presenting cauda tubules with less sperm in the lumen. In the same way, (K) BPS (5 mg/kg/day) exposed group, presenting normal caput tubules like in the control, (L) BPS (25 mg/kg/day) exposed group, presenting cauda tubules with sperm filled lumen, (M) BPS (50 mg/kg/day) exposed group presenting cauda tubules with less sperm in the lumen. H&E (40x).

DISCUSSION

A large number of studies has reported the adverse effects of BPA involvement in many chronic diseases. Therefor the increasing concerns of environmental security agencies and government has led to the development of some potential substitutes for BPA such BPB, BPF and BPS. There is very little data available on the different doses of BPA and its analogues BPB, BPF and BPS that have already being used in many of the daily use items in the market. This study was designed to analyze the effects of different doses of BPA analogues on the development of male reproductive system in male rats, in comparison with the effects of BPA in the same range concentrations. In this study we exposed male rats to different concentrations of BPA and its analogues BPB, BPF and BPS subcutaneously during neonatal period of life. In which we showed that BPA analogues have the same deleterious properties as BPA and are as toxic as BPA on the reproductive system of male rats.

In our present study we observed that male reproductive system is sensitive to different concentrations of BPA and its analogues by showing effects on the different cell types and hormones. However, these doses during the neonatal life exhibited different effects in adult period of life on having adverse effects on the different reproductive parameters of reproductive system. The different doses of BPA and its analogues were selected from different available data of low and high dose exposure to male rats during neonatal period of time (Fernández *et al.*, 2010, Kinch *et al.*, 2015).

In the present study animals neo-natally exposed to the higher doses of BPA and its analogues were observed with an increase in their final body weight. Which are in relation with previous studies were an increase was observed in adipogensis when human preadipocytes were exposed to different concentrations of BPA analogues and similar results were found in rats exposed to different concentrations of these bisphenols in their prenatal life (Boucher *et al.*, 2016a, Salian *et al.*, 2011, Del Moral *et al.*, 2016, Ahmed and Atlas, 2016). In our present study we observed reduction in the gonadal somatic index of adult male rats to BPA and its analogues which suggest an interesting finding that high doses of these bisphenols are involved in hazardous effects on the development of reproductive system. GSI is also considered an important parameter in determining the time of gonadal maturation (Ahsan *et al.*, 2018a, Hachfi *et al.*, 2012, Naderi *et al.*, 2014).

There were also estrogenic mode of actions observed of BPA and its analogues like inducing reduction in weight of seminal vesicle and epididymis which has previously also been characterized for some of these analogues by involving in reducing the weight of reproductive organs (Zhang *et al.*, 2017b, Cagen *et al.*, 1999a, Zatecka *et al.*, 2013). Reproductive organs possess both estrogen and androgen receptors and reduction in weight of these organs lead into disrupted receptors for these hormones which in result lead into poor reproductive system (Sonnenschein and Soto, 1998, Pelletier *et al.*, 2000, Lubahn *et al.*, 1993).

It has been observed previously that neonatal exposure to different concentrations of BPA and its analogues lead to reduced testosterone production in adulthood which suggest that these chemicals androgenic and estrogenic effects are involved in disturbed hormonal levels (Nakamura *et al.*, 2010, Feng *et al.*, 2012, Jin *et al.*, 2013). In our study we also observed reduced levels of plasma testosterone, LH and FSH while estrogen levels were high after neonatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS which also suggest the link of both androgenic and estrogenic mode of actions of these chemicals on the reproductive system. In the past there have been several studies supporting our current results in some of these bisphenols in both *in vivo* and *in vitro* models (Lee *et al.*, 2003, Williams *et al.*, 2001, Wetherill *et al.*, 2007, Wilson *et al.*, 2008, Earl Gray Jr *et al.*, 2006).

BPA and its analogues BPB, BPF and BPS suggest its endocrine disrupting mode by altering LH and FSH concentrations in animals treated with different concentrations as compared to the control. Where in male synthesis of androgens is linked with the normal spermatogenesis. The alerted androgens support the altered histopathological result suggesting that alterations in the androgen levels lead into alterations in the normal spermatogenesis in the testis.

In the previous studies it was reported that oxidative stress induced by BPA and some of its analogues resulted in disturbed hormones in the different organisms (Moghaddam *et al.*, 2015, Hassan *et al.*, 2012, Feng *et al.*, 2016, Naderi *et al.*, 2014, Yang *et al.*, 2017a). In different studies previously, it was also reported that BPA and BPS exposure lead to oxidative stress in the peripheral blood mononuclear cells in testis and also lead to lipids and protein degradation *in vitro* (Michałowicz *et al.*, 2015, Ullah *et al.*, 2017, Ullah *et al.*, 2016, Mokra *et al.*, 2015). The results of our study about inhibition of testosterone and anti-androgenic effects of these chemicals are in line with studies of (Rochester and Bolden, 2015, Molina-Molina *et al.*, 2013). Testosterone reduced concentrations might be a result of suppression of GnRH transcripts in the

hypothalamus which also suggest that suppressed GnRH lead to reduced gonadotropin secretion (Ji *et al.*, 2013, Roelofs *et al.*, 2015). However, increased estrogen levels seem to be due to estrogenic mode of action of BPA and its analogues BPB, BPF and BPS.

Besides, the reduction in the LH and FSH levels we observed reduced testosterone concentrations, reduced DSP and number of sperm in epididymis exposed to different concentrations of BPA and its analogues BPB, BPF and BPS. Similarly, the reproductive organs weights were also reduced in different concentrations exposed groups to BPA and its analogues BPB, BPF and BPS. Our results are in accordance with the different studies were BPA and its analogues have been observed to result in increase in the adipogenesis (Somm *et al.*, 2009, Héliès-Toussaint *et al.*, 2014, Ahmed and Atlas, 2016). BPA and its analogues have also been observed to be associated with obesity and high fat in the different organs in the body (Boucher *et al.*, 2016b, Del Moral *et al.*, 2016, Vom Saal *et al.*, 2012, Somm *et al.*, 2009).

It has also been observed that BPA and some of its analogues are also involved in alteration of the antioxidant enzymes structure by inducing toxicity in different organs of the reproductive system (Ullah *et al.*, 2018, Wu *et al.*, 2011). Different *in vitro* and *in vivo* studies have shown BPA and some of its analogues exposure in inducing antioxidant enzymes activity, increasing ROS concentrations, reduction in DSP and alterations in the tubules of somniferous tubules (Wu *et al.*, 2011, Ullah *et al.*, 2018, Ullah *et al.*, 2017, Ullah *et al.*, 2016).

Different sperm parameters were observed in the present study showing significant reduction in the percentage of motile sperms in the high dose groups of BPA and its analogues BPB, BPF and BPS. There were also fewer sperms observed in the caput and corpus epididymis and the number of DSP was also reduced in the epididymis in some of the treated groups. Our current results are in accordance with some of the previous studies were neonatal exposure to BPA led to arrest in the number of spermatogonial cells in the testicular tissues (Ma *et al.*, 2017, Tootian *et al.*, 2016, Xie *et al.*, 2016, Al-Hiyasat *et al.*, 2002, Okada and Kai, 2008). It has been observed from these results that BPA analogues not only lead to reduction in the number of sperm production but also lead to disturbance in the cauda epididymis environment by reducing the viability and motility of sperms present in there.

In our present study planimetry and histological observations showed significant reduction in epithelial height, and seminiferous tubules diameter. There were also reduced number of spermatids, spermatogonia and spermatocytes observed in some of the treated groups of BPA

and its analogues after neonatal exposure in the adult male rats. The main changes observed were the reduced size of seminiferous tubules and large spaces in the interstitium and lumen after BPA and its analogues exposure. Our current results are in accordance with some of the previous studies which have shown that BPA and some of its analogues led to affected morphology of nucleus, DNA damage and alterations in the antioxidant enzymes profile (Ullah *et al.*, 2016, Li *et al.*, 2014b, Kourouma *et al.*, 2015, Sidorkiewicz *et al.*, 2017, Ullah *et al.*, 2018). In the present study the steroid hormone levels in the treated groups were altered after exposure to BPA and its analogues BPB, BPF and BPS which led to poor spermatogenesis. The alterations in the testis and reproductive system are dependent upon the exposure period duration in the neonatal rats. In the current study we also observed spermatogoneal arrest which is an important stage in the normal spermatogoneal process which needs to be investigates after short interval of exposure to these endocrine disruptors. Though there were no alterations observed in the morphometry of the different parts of epididymis where there were prominent changes in the diameter of different cells after exposure to BPA and its analogues BPB, BPF and BPS. The number of sperm was also less as compared to the control when different sections of epididymis were observed suggesting thin layer of sperm in the tubules.

Conclusions

In the present study we observed that exposure to different concentrations of BPA and its analogues BPB, BPF and BPS during neonatal period bring about prominent changes in the endocrine system of male rats by altering hormonal profile and affecting sperm parameters. In the present study we also observed that reduction in the viability and motility of sperms and arrest in the degree of spermatogoneal cells after neonatal exposure to different concentrations of BPA and its analogues BPB, BPF and BPS.

GENERAL DISCUSSION

BPA has had a very long history as a monomer it is produced in large quantity and for the production of epoxy resins and polycarbonate plastics. BPA is used in the manufacturing of food cans, baby bottles and water pipes (Rubin, 2011). It is also found in many consumer products these days such as dental composites, metal cans linings, thermal paper and food containers. Due to its high use in many consumer products BPA can leach from consumer products under acidic/basic conditions, high temperature and has already shown many sources of human and animals exposure mostly via inhalation, dermal routes and digestion (Vandenberg *et al.*, 2010a, Vandenberg *et al.*, 2010b). Thus the health concerns regarding BPA are increasing due to its widespread human exposure (Calafat *et al.*, 2008, Qi and Zhang, 2011). BPA phenolic structure allows it to interact with estrogen receptors and act as agonist or antagonist via signaling pathways of endocrine receptors (Ma and Sassoon, 2006). Therefore, it has been able to play role in the pathogenesis of many endocrine disorders including female and male fertility and many metabolic disorders and hormone-dependent tumors such as prostate and breast cancer (Diamanti-Kandarakis *et al.*, 2009). There are many routes of BPA exposure such as oral, transdermal and inhalation. BPA main source of exposure includes healthcare equipment, thermal papers, toys, food packaging, indoor dust and infant feeding bottles (Geens *et al.*, 2012a). Among the main sources of BPA exposure, there is quite a big contribution of canned food stored in boxes either made or coated with BPA, meat and eggs of animals exposed to water with BPA (Huang *et al.*, 2012, Van Landuyt *et al.*, 2011, Oldring *et al.*, 2014). BPA exposure in human studies show its association with reduced ovarian response, implantation failure, miscarriages, reduced male sexual functions, reduced sperm quality, altered sex hormones, altered liver functions and oxidative stress (Richter *et al.*, 2007, Bonefeld-Jørgensen *et al.*, 2007, Moriyama *et al.*, 2002, Vom Saal *et al.*, 2007). BPA has also been associated with abnormal gestation time, reduced birth weight, increase male genital abnormalities and obesity in children (Rubin *et al.*, 2001, HIROI *et al.*, 2004, Soto *et al.*, 2008). It has also been observed to altered behavior and neurodevelopment in children. The above all abnormities and complacencies have been supported by both the *in vitro* and *in vivo* studies (Midoro-Horiuti *et al.*, 2010, Miyawaki *et al.*, 2007, Toyama *et al.*, 2004, Berger and Shaw, 2008, Chitra *et al.*, 2003, Williams *et al.*, 2001).

BPB is an analogue of BPA used for the production of phenolic resins (Chen *et al.*, 2002b). BPB has also been found in 21% of food and beverages as it leaks very similar to BPA (Grumetto *et al.*, 2008). There have also been studies were BPB has been found in the endometriosis of women (Cobellis *et al.*, 2009, Mendes, 2002). In the canned beverages BPB levels have been found high in many brand samples across the globe (Cunha *et al.*, 2011, Cunha *et al.*, 2012, Cunha and Fernandes, 2013). There have also been several studies which have shown that BPB has both estrogenic and anti-androgenic properties (Ike *et al.*, 2006, Kitamura *et al.*, 2005, Yoshihara *et al.*, 2001).

BPA another analogue BPF contains two phenol rings similar to BPA. BPB has already started gradually replacing BPA by having many applications in manufacturing industry of polycarbonates plastics and epoxy resins (Yamazaki *et al.*, 2015, Lee *et al.*, 2015, Yu *et al.*, 2015, Molina-Molina *et al.*, 2013). In the recent years there have been several studies which have shown BPF residues in food containers, epoxy resins and water pumped by pipes made of BPF (Stroheker *et al.*, 2004, Goodson *et al.*, 2002, Usman and Ahmad, 2016). BPF has also been detected to a toxic concentration in the food stuff like fish, sea food, meat products, beverages and vegetables (Gallart-Ayala *et al.*, 2011a, Yamazaki *et al.*, 2015, Lee *et al.*, 2015, Rochester and Bolden, 2015). BPF has also been observed to induce oxidative stress, induction in the lipid peroxidation and increasing ROS levels (Audebert *et al.*, 2012). It has also be found to have genotoxicity and endocrine toxicity (Cabaton *et al.*, 2009).

BPA family another member BPS has two phenolic groups linked by sulphur dioxide group. In 1869 it was firstly introduced into the environment and later in 2006 it was used in the cash register receipts (Glausiusz, 2014). When BPA was banned in several daily use items so the plastics industry switched to the so called safer analogues of BPA like BPB, BPF and BPS and the use of BPS started gaining momentum in the production of baby bottles, thermal papers and epoxy resins (Becerra and Odermatt, 2012, Becerra and Odermatt, 2013, Grignard *et al.*, 2012, Liao *et al.*, 2012c, Rochester and Bolden, 2015, Liao *et al.*, 2012a, Rosenmai *et al.*, 2014). BPS has toxic effects in several studies it has been observed to have hormonal potencies similar to BPA and it exerts acute cytotoxicity by inducing DNA damage (Chen *et al.*, 2002b, Ji *et al.*, 2013, Liao *et al.*, 2012c, Flint *et al.*, 2012).

BPA and its analogues have been observed to have toxic effects by including cytotoxicity, genotoxicity and endocrine disruption. There have been studies which have also shown very

clearly that those BPA analogues like BPB, BPF and BPS have potency similar to BPA for estrogenic, antiestrogenic, androgenic and antiandrogenic activities (Rochester and Bolden, 2015). In a study on zebrafish when BPS exposure significantly reduced the number of eggs and decreased the gonadosomatic index (Ji *et al.*, 2013). When F1 zabrfish embryos were exposed to BPS it was observed that the hatchability decreased and malformation increased. Exposure to BPA and its analogues has also led to disturbed development of the offspring and also caused disturbance in the feedback regulatory index at the hypothalamus pituitary gonadal (HPG) axis. Hormonal balance and reproductive potentials were also impaired after exposure to BPA and its analogues at the developmental level (Ji *et al.*, 2013, Naderi *et al.*, 2014). In another study when zebrafish embryos were exposed to BPA and BPS it was observed that neurogenesis increased at the level of hypothalamus and also resulted in the hyperactive behavior in the later stages (Kinch *et al.*, 2015). Another study showed that exposure to BPA and BPS treatment to mouse 3T3-L1 adipocytes increased glucose uptake and leptin production (Héliès-Toussaint *et al.*, 2014). These all findings at this stage suggest that both BPA and BPS are involved in the obesity, metabolic pathways disturbances and different reproductive mechanisms (Ma *et al.*, 2015). BPA analogues are also involved in the toxicities quite similar to BPA because these all analogues poses similar mode of actions raising the safety concerns on the applications of BPA replacements (Chen *et al.*, 2002b, Yokota *et al.*, 2008, Ullah *et al.*, 2018). BPA analogues toxicity studies remain remarkably limited so far regarding mode of actions and quantitative toxicity in both *in vivo* and *in vitro* studies.

In the a number of our studies we have also found that BPA and it's so called safe analogues lead to oxidative stress in the shape of reactive oxygen species and lipid per oxidation in the different reproductive tissues and also sperm. In both *in vitro* and *in vivo* studies we observed oxidative stress in the reproductive organs of male rats after exposure to BPA and its analogues BPB, BPf and BPS. Higher levels of oxidative stress in *in-vivo* and *in-vitro* studies were observed in the higher exposed doses of BPA and its analogues. We have also observed in the previous studies that BPA and some of its tested analogues are involved in inducing oxidative stress in the reproductive organs (Ullah *et al.*, 2016, Yang *et al.*, 2016, Ahmed *et al.*, 2018, Kourouma *et al.*, 2015). It was found out that these BPA analogues induce oxidative stress in the different cell lines *in vitro* and also lead into DNA damage in *in vivo* studies (Zhang *et al.*, 2018b, Geetharathan, 2016, Moustafa and Ahmed, 2016).

In all the *in vivo* studies and *in vitro* studies comparative exposure to BPA and its analogues BPB, BPF and BPS caused reduction in the testosterone concentrations and also significant reduction in the LH and FSH concentrations as compared to control. In the *in vitro* study, comparative exposure to BPA and its analogues BPB, BPF and BPS reduced testosterone secretions in the testis of rats. Along with the *in-vitro* in the *in vivo* studies both chronic and sub-chronic significant reduction was observed in the steroid hormones after exposure to BPA and its analogues BPB, BPF and BPS. Furthermore, significant reduction was also obvious in the rats exposed neonatlly to BPA and its analogues BPB, BPF and BPS. However, there was no significant difference observed in the steroid hormones of rats exposed to BPA and its analogues during their prenatal life in comparison with the neonatal life.

The results of present studies indicate the endocrine disrupting potential of BPA and its analogues BPB, BPF and BPS in mammals, suggesting that BPA analogues induce estrogen levels in the males and can inhibit testosterone secretions by interrupting gonadotropins secretions from pituitary, depending upon the dose levels and duration of time for which the animals were exposed. Higher concentrations of BPA and its analogues BPB, BPF and BPS were found effective for inhibiting gonadotropins levels and testosterone secretions in male rats (Wu *et al.*, 2011, Maćczak *et al.*, 2017, Ullah *et al.*, 2016, Alexander *et al.*, 1988, Ahmed *et al.*, 2018, Rosenmai *et al.*, 2014, Feng *et al.*, 2016).

In our comparative studies with BPA and some of its analogues we also found out that *in vivo* exposure disturbed the sperm parameters in testes and also led to disturbance in the different kind of cells inside the reproductive tissues both testis and epididymis. Spermatogenesis arrest and reduced daily sperm production was also observed in the *in vivo* studies which means that exposure to different concentrations of BPA and its analogues lead to disturbed gonadotropins which in return lead into disturbed histological changes. Most of the disturbed histological changes and hormonal imbalances were observed in the higher exposed groups in our studies.

In previous studies, it was observed that BPA and its analogues BPB, BPF and BPS induced apoptosis, disturbances in calcium ions in the cytosol, chromatin condensation, activated caspases and reduced membrane potentials of mitochondria, suggesting the necrosis and apoptosis inducing potentials of these compound, reduced testosterone production in fetal testis *in vitro*, disturbance of GSI, increase in estradiol concentrations, reduction in plasma testosterone

concentrations, change in GnRH transcripts expressions and many more complacencies in the different animals (Rivas *et al.*, 2002, Maćczak *et al.*, 2016, Rochester and Bolden, 2015, Mokra *et al.*, 2015, Fic *et al.*, 2013a, Michałowicz *et al.*, 2015, Katoh *et al.*, 2004, Yang *et al.*, 2017a, Mu *et al.*, 2018a, Yang *et al.*, 2017b, HASSANIN *et al.*, 2002).

BPA analogues BPB, BPF and BPS toxic effects in the reproductive organs highlight their potential toxicity in male rats, now there is a need of comprehensive studies to evaluate their potentials reproductive toxicity in the other mammal species. Several of our results from these comparative studies show that exposure to these analogues at any stage of life can cause alterations in the spermatogoneal cells which can lead into disturbed reproductive system in both human and animals increasing the chances of infertility.

GENERAL CONCLUSIONS

In the final conclusion of our current series of studies we have come to observe that exposure to BPA and its analogues BPB, BPF and BPS alter the reproductive system of male rats by inducing oxidative stress in the testicular tissues which affects directly or indirectly the viability and motility of sperms. In sub-chronic and chronic studies, we also observed disturbed reductive functions in male rats which give an indication of adverse effects of these analogues for long period exposure in human as occupational period. Arrest of spermatogenesis at spermatogonal cells stage was also observed in the developmental exposure to BPA and its analogues BPB, BPF and BPS. There were also endocrine disrupting potentials of these BPA analogues observed in the disturbed concentrations of testosterone, LH, FSH and estradiol which also suggest that exposure to higher concentrations of BPA and its analogues BPB, BPF and BPS can also lead to adverse reproductive health effects in both human and different animal species. However, our current findings open new sights into reproductive toxicity induced by these BPA analogues which the manufactures claim to be safe enough need further studies to clarify the mechanism of actions under which the toxic effects of these endocrine disrupting chemicals act on the other organs of our body.

Summary of the five sets of experiments on the comparative study of BPA and its analogues BPB, BPF and BPS

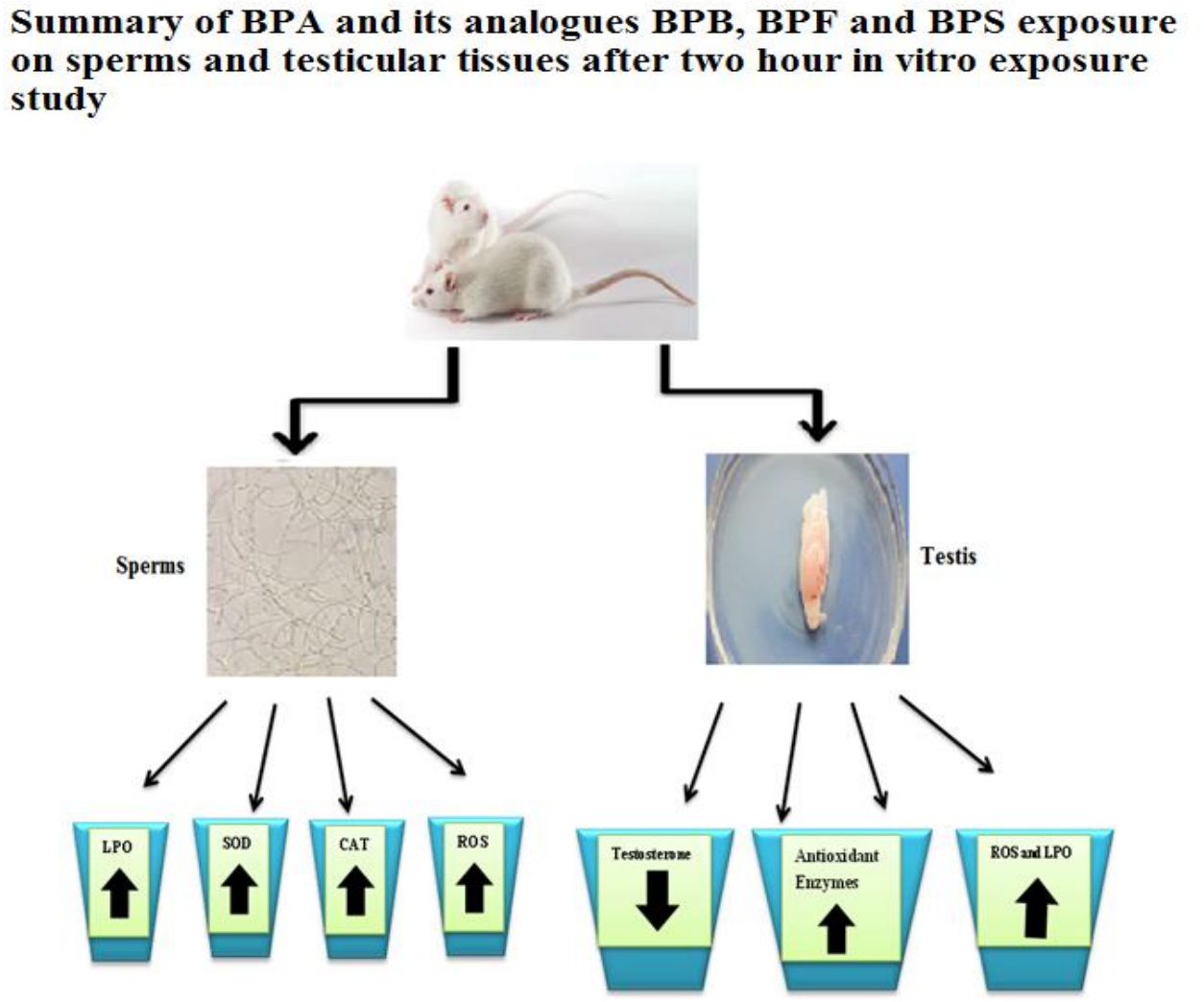

Fig 20. *In vitro* exposure of different concentrations of BPA and its analogues BPB, BPF and BPS on the sperm and testicular tissues of adult male rats. BPA and its analogues exposure reduced testosterone secretions and induced oxidative stress.

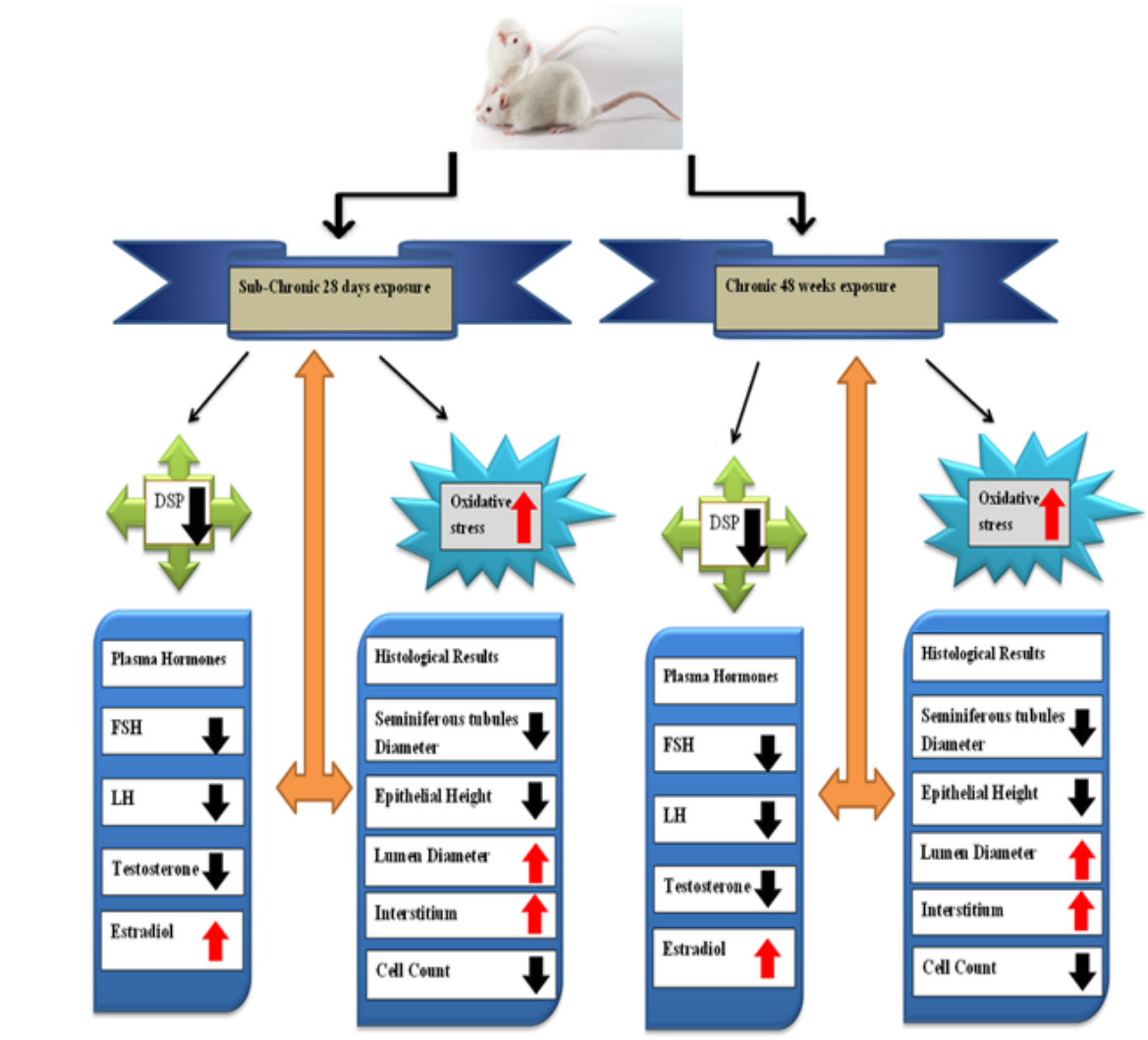

Fig 21. ***In vivo*** **sub-chronic and chronic effects of different concentrations of BPA, BPB, BPF and BPS on the different parameters of hypothalamo-pitutary-testicular (HPT) axis of male rats. The above schematic representation shows that BPA and its analogues BPB, BPF and BPS exposure in both sub chronic and chronic study can alter hormones concentrations, leads to oxidative stress and cause morphological changes in the number of different cells population in the testis and epididymis.**

Summary of BPA and its analogues BPB, BPF and BPS pre-natal and neo-natal exposure on the development of male rats

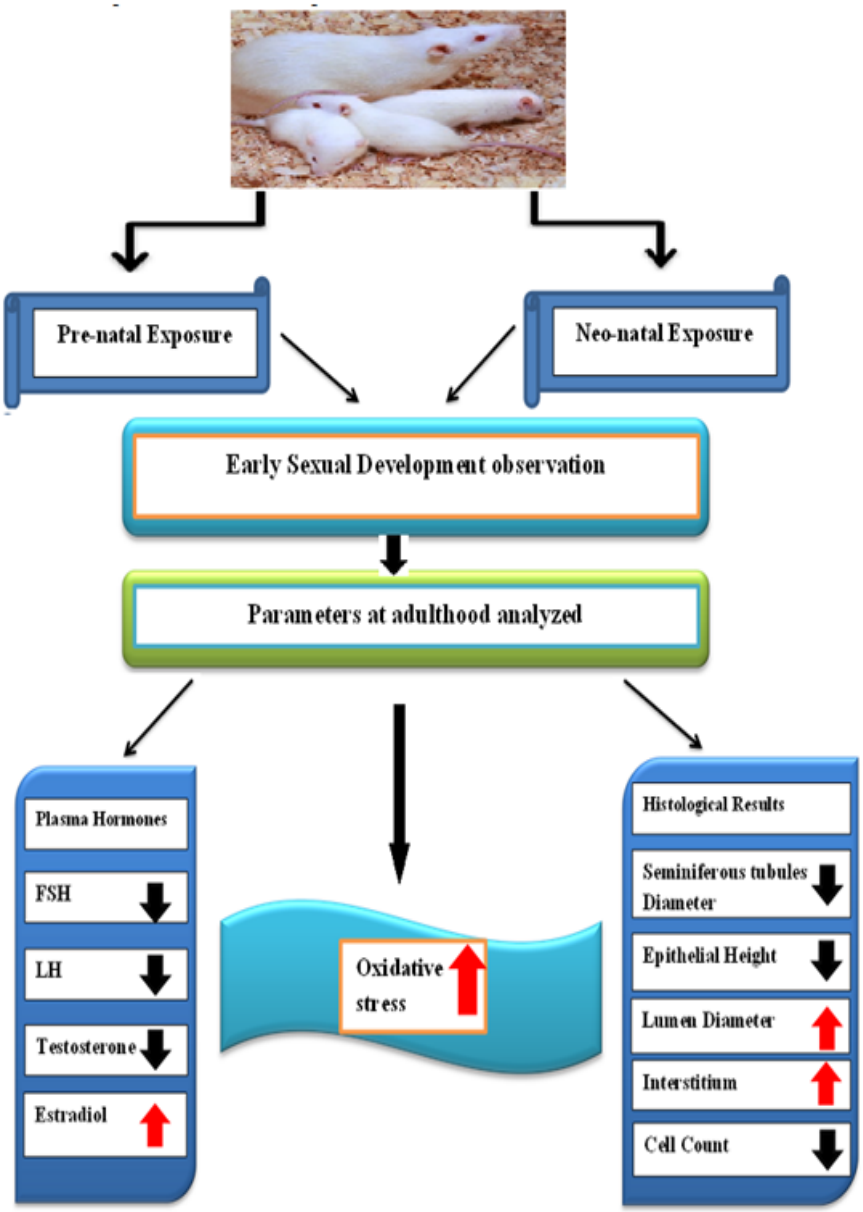

Fig 22. Schematic representation showing hazardous effects of BPA and its analogues BPB, BPF and BPS on the reproductive system during prenatal and neonatal exposure.

FUTURE PERSPECTIVES

The widespread exposure of BPA has led into a large number metabolic and coronary diseases (diabetes, obesity and reproductive disorders) where in some countries restrictions have been imposed on the use of BPA and a shift in the use of its analogues has already started across the globe. The production of BPA analogues is also at great rise and it is taught that it will become a serious contamination due to their presence being felt in various environmental compartments as well as food and beverages. The rise in the daily use items has kept both occupational and general population under risk of exposure. BPA analogues have similarity in structure with BPA and have also been observed to act via endocrine disruption like BPA. This has left us with lots of questions that whether this BPA analogues shift is safe or more threatening than BPA exposure. The present series of studies has raised concerns that these BPA analogues BPB, BPF and BPF are more threatening than BPA in terms of oxidative stress and reproductive toxicity. BPA has been studied in great detail regarding reproductive toxicity but data regarding BPA analogues is scarce and lots of experimental work has to be carried out. A data bank is needed regarding BPA analogues toxicity and comparative toxicity studies shall be taken out regarding BPA and its analogues.

- BPA analogues BPB, BPF and BPS interactions with different receptors (ER α, β) at cellular and molecular level needs to be determined.
- BPA and its analogues interactions with EDCs and life style needs of diet, living and using shall be analyzed so that its presence in different items and its concentrations in different population be determined.
- Metabolic, cellular and endocrine pathways through which these analogues act in different organs and it different cells shall be analyzed.
- BPA and its analogues interaction with different cells and receptors shall be check on the molecular level.
- General and occupationally exposed population shall be analyzed for the detection of the different BPA analogues in serum, urine and other bodily fluids.
- The results of the current study have somehow covered BPA and it’s so called safe analogues BPB, BPF and BPS reproductive toxicity, endocrine disruption and oxidative stress and further experimental work is required to understand the cellular and molecular

mechanism through which the mode of action of these chemicals be understood on the different experimental animals.

- BPA and its analogues BPB, BPF and BPS potential toxic effects are urgently required in order to assess the safety of these chemicals and information is needed regarding the toxic impurities of these analogues byproducts in BPA free items and baby feeding bottles.
- BPA and its analogues are suspected to have adverse effects on the development of nervous system and reproductive system and longtime exposure studies shall be carried out in animals and humans.
- Finally, the further investigation of BPA and its analogues BPB, BPF and BPS shall be carried out on their metabolites occurrence in human urine, serum and body fluids and these metabolites bioavailability in the environment shall be carried out.

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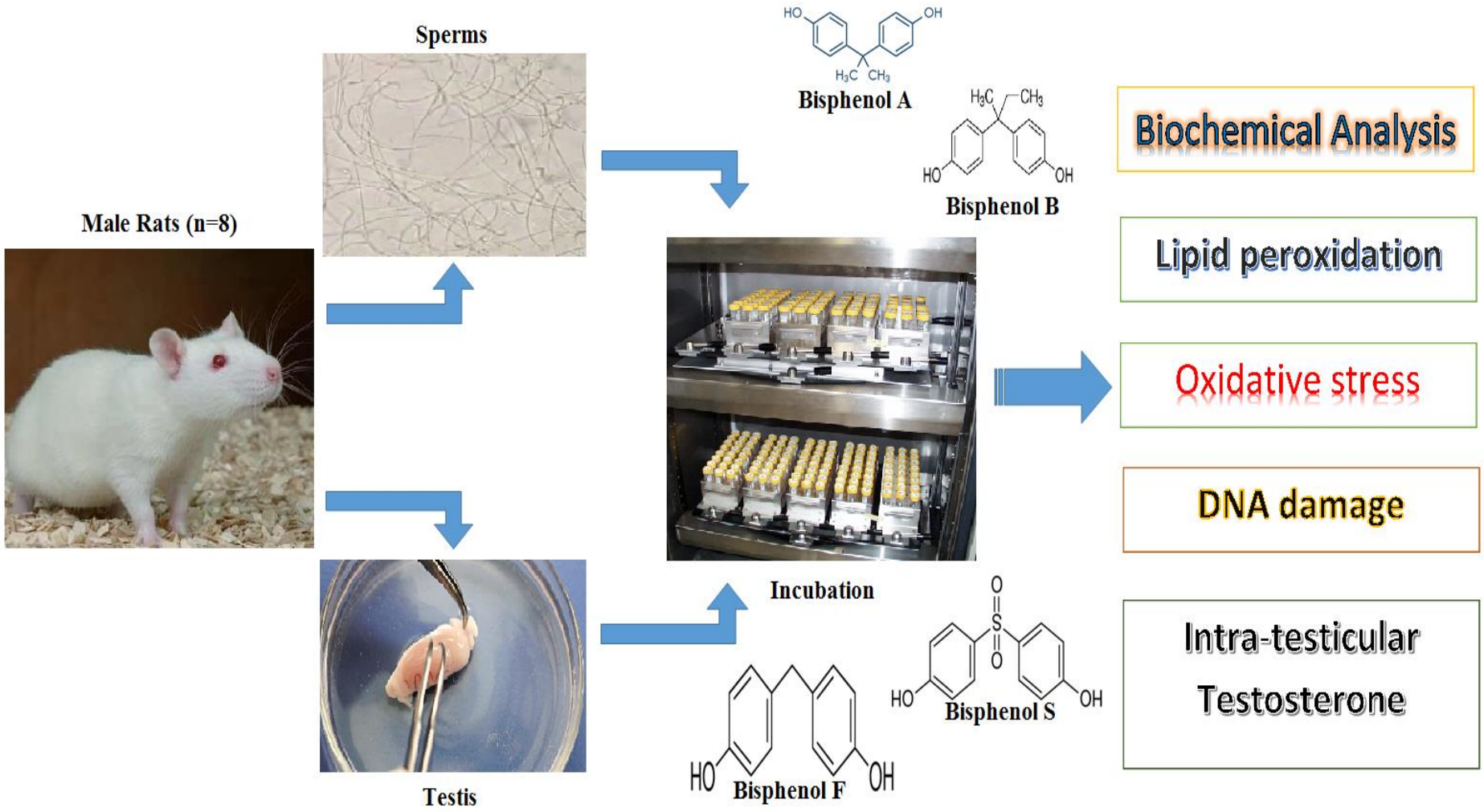
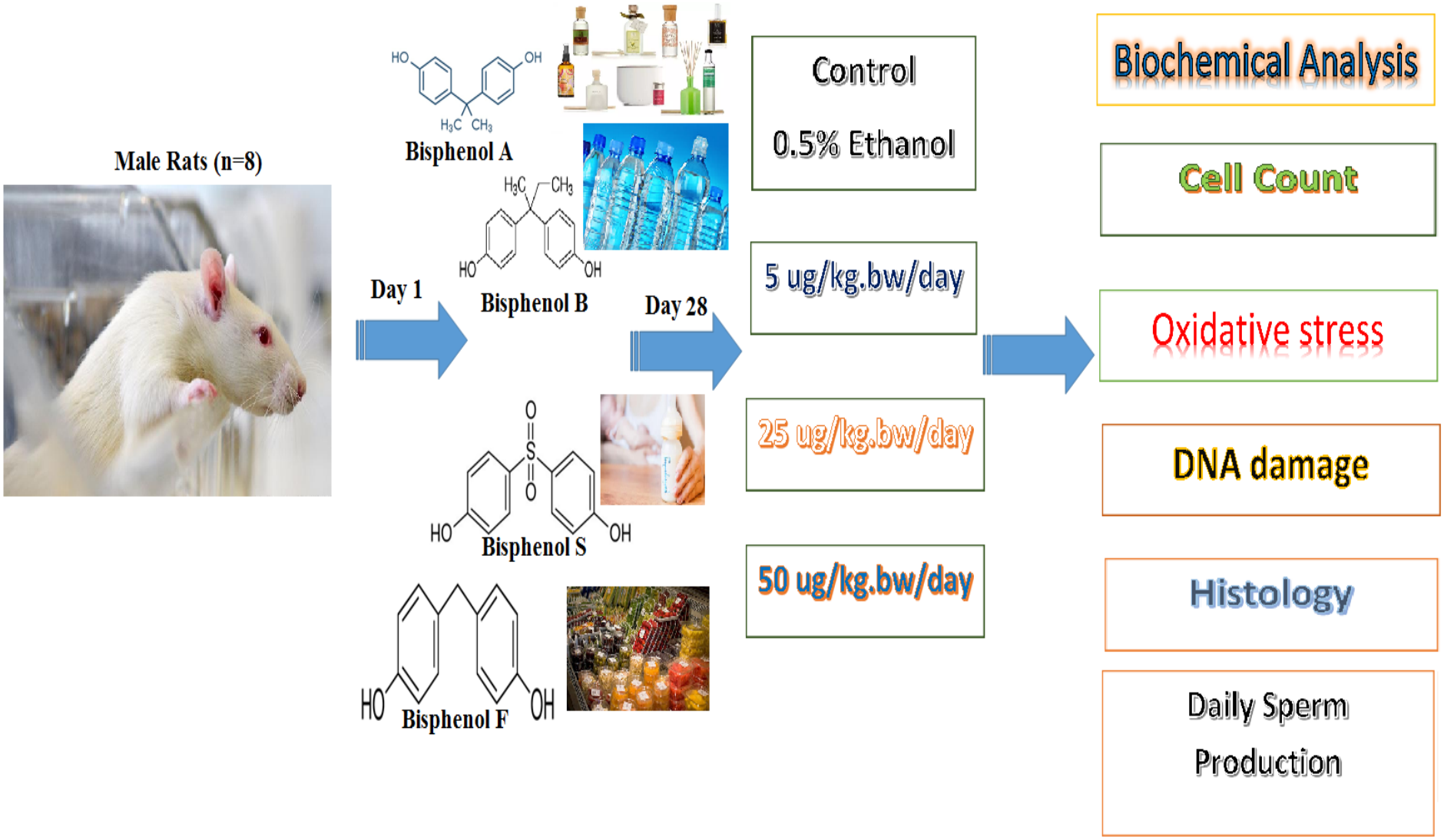
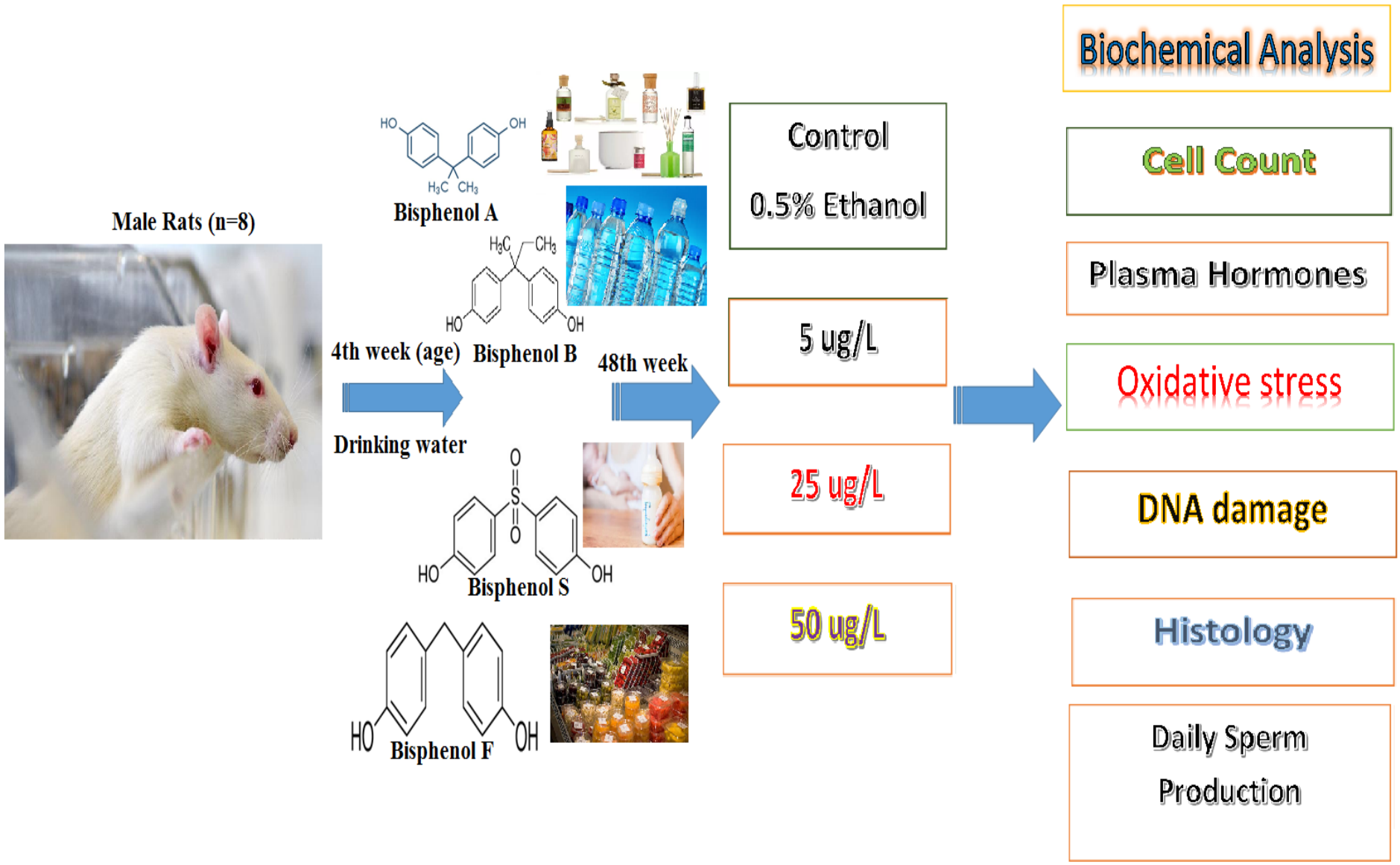


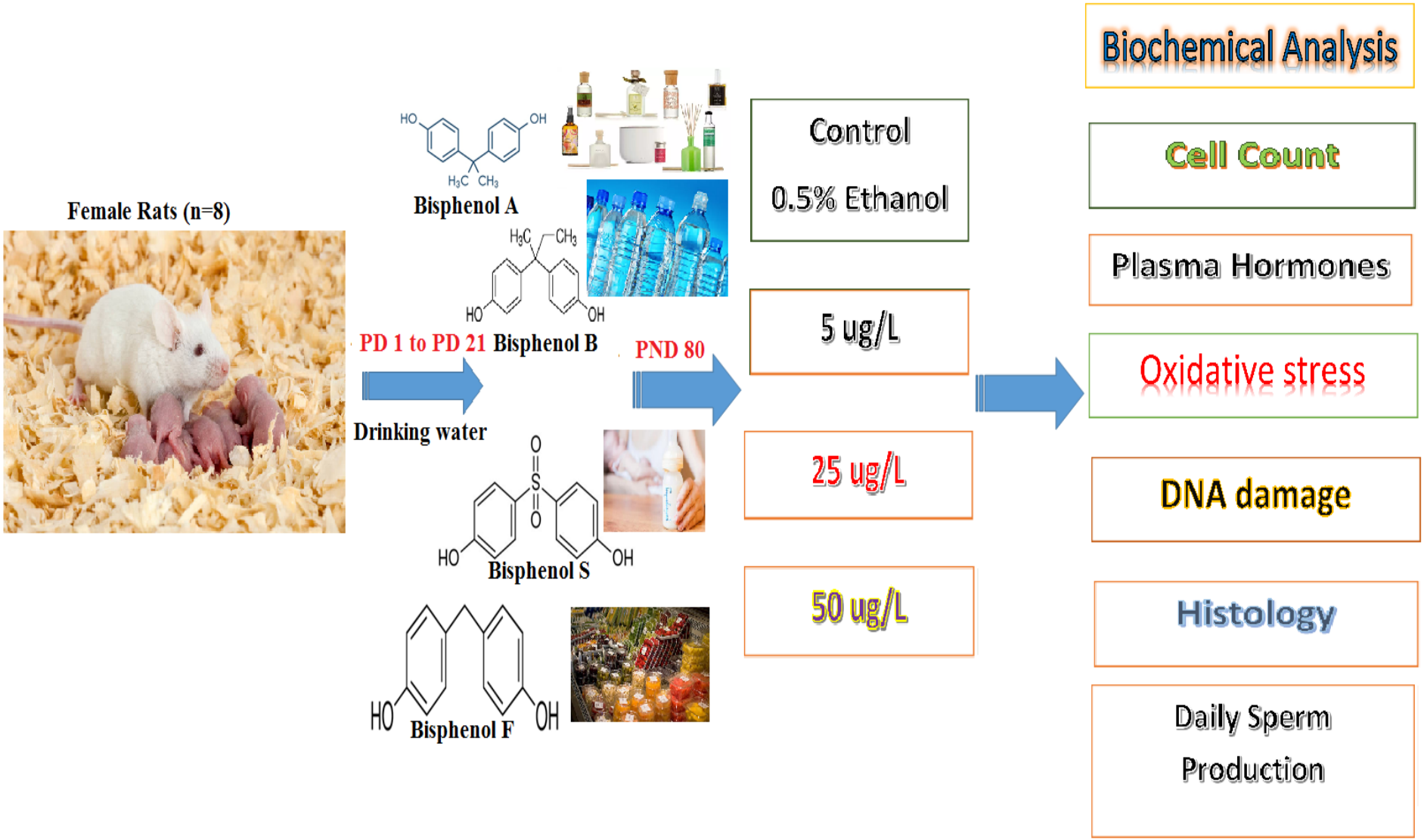
Schematic experimental design of bispehol A (BPA) and its analogues bispehol B (BPB), bisphenol F (BPF) and bisphenol S (BPS) exposure to testis after 2 hour incubation.



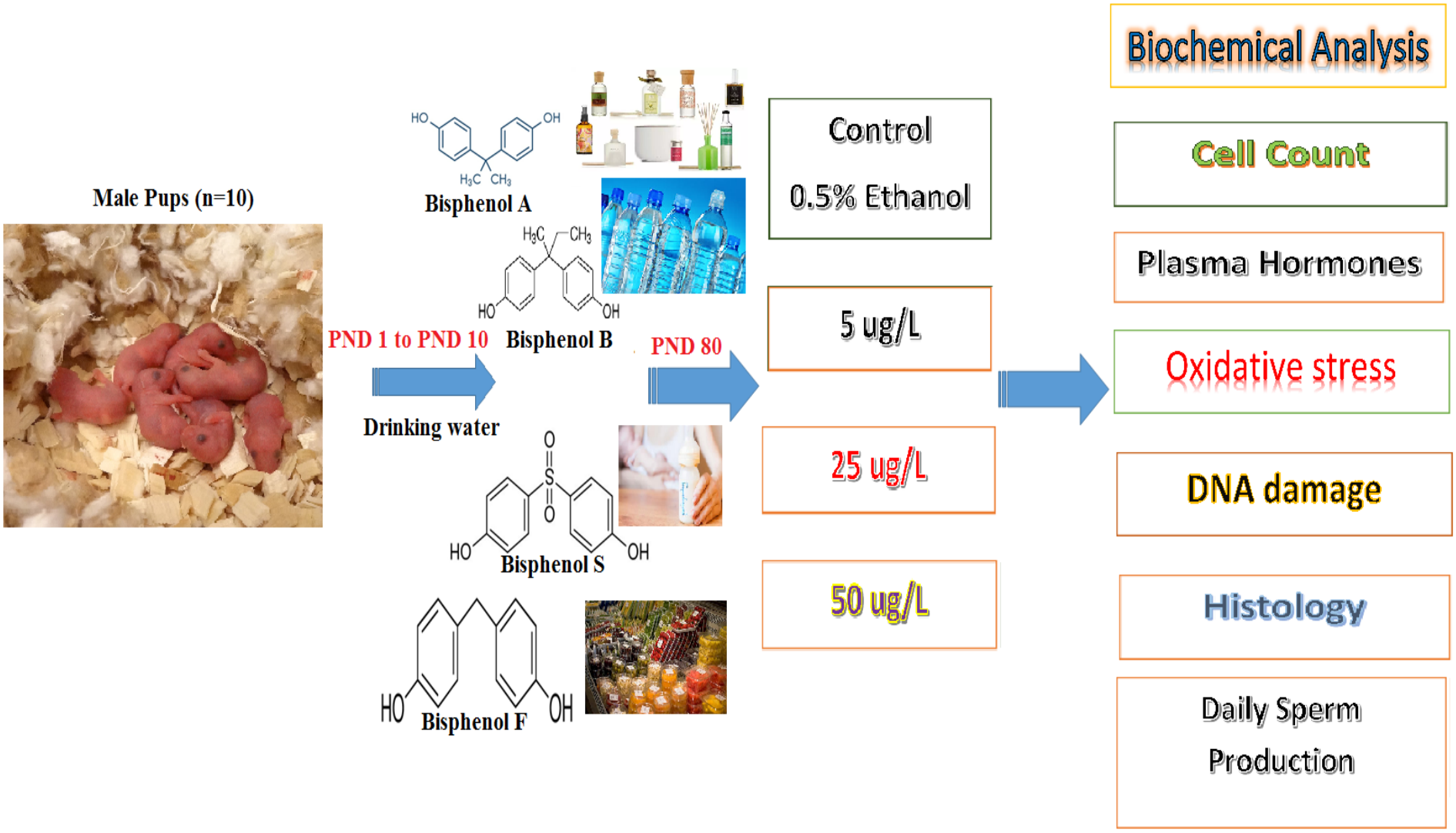
Schematic representation of sub-chronic exposure to bispehol A (BPA) and its analogues bispehol B (BPB), bisphenol F (BPF) and bisphenol S (BPS) in adult rats.



Schematic representation of the experimental design for chronic exposure to bispehol A (BPA) and its analogues bispehol B (BPB), bisphenol F (BPF) and bisphenol S (BPS) in rats.



Schematic representation of the experimental design for prenatal exposure to bispehol A (BPA) and its analogues bispehol B (BPB), bisphenol F (BPF) and bisphenol S (BPS) in rats.



Schematic representation of the experimental design for neonatal exposure to bispehol A (BPA) and its analogues bispehol B (BPB), bisphenol F (BPF) and bisphenol S (BPS) in rats.

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Bisphenol A and its analogs bisphenol B, bisphenol F, and bisphenol S: Comparative in vitro and in vivo studies on the sperms and testicular tissues of rats

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HIGHLIGHTS

- Comparative toxicity effects of BPA and its analogs BPB, BPF, and BPS on the reproductive system of male rats.
- In vitro study was conducted with cultured cells of the rat testicular tissue, BPA, BPB, BPF, and BPS induced oxidative stress.
- BPA and its analogs BPB, BPF, and BPS reduced plasma and intratesticular testosterone concentrations.

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ABSTRACT

Bisphenol A (BPA) is used as the main component of many consumer products such as infant's feeding bottles, coatings of beverages, and food cans. BPA can migrate into the environment, and it has been detected in the saliva, blood, and food. BPA leakage from many consumer products resulted in a ban on its use in many countries where alternatives to BPA were introduced into the market. BPA alternatives such as bisphenol B (BPB), bisphenol F (BPF), and bisphenol S (BPS) have a similar chemical structure and binding ability for estrogen receptor (ER), which shows toxicological effects in animals. In the present study, comparative effects of exposure to BPA and its analogs BPB, BPF, and BPS on testosterone concentration in the rat testis were evaluated by in vitro and in vivo approaches in which oxidative stress markers and antioxidant enzyme activities in reproductive tissues were determined. In the in vivo study, male rats were exposed to different concentrations of BPA and its analogs BPB, BPF, and BPS (5, 25, and 50 mg/kg/day) for 28 days. In the in vitro exposure study, antioxidant enzyme activities and oxidative stress markers were induced in the testes, whereas testosterone production was reduced. In the in vivo exposure study, we observed that antioxidant enzyme activities and protein content were reduced, whereas reactive oxygen species and lipid profile were increased in the treated groups compared to the control group. The present comparative study on BPA and its analogs, namely, BPB, BPF, and BPS suggests the toxic effect of these chemicals on the testes and spermatogenesis, and we also observed that these chemicals induce oxidative stress in the reproductive tissues of male rats.

1. Introduction

Bisphenol A (BPA; 4,40-dihydroxy-2,2-diphenylpropane) has had a very long story in the history of sciences. In 1936, the estrogenic properties of BPA were reported in the female reproductive system of rats (Dodds and Lawson, 1936), and BPA was introduced into the industry for polymer (epoxy resins, polycarbonate, and certain plastics) synthesis (Scippo, 2011). BPA was used as the main component of many consumer products such as infants' feeding bottles, coatings of beverages and food cans,

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medical devices, and dental sealants (Scippo, 2011; Huang et al., 2012). Depending upon the exposure to temperature and pH, BPA can migrate into the environment, and it has also been detected in the saliva, blood, and food (Ahn et al., 2008; Calafat et al., 2008, 2009; Braun et al., 2011; Scippo, 2011; Van Landuyt et al., 2011). As a weak estrogen, bisphenols have two OH and benzene rings, which fit into the binding pocket of the estrogen receptor (ER) (Kuiper et al., 1998; Grignard et al., 2012), and the binding affinity of BPA makes it a classical ligand for both ER α and ER β, and this also increases its estrogen potency (Wetherill et al., 2007; Alonso-Magdalena et al., 2012).

In response to American Chemistry Council in 2012, Food and Drug Administration (FDA) banned the use of BPA in sippy cups, infants' bottles, and thermal receipt papers. This ban resulted in the introduction of BPA alternatives such as bisphenol B (BPB), bisphenol F (BPF), and bisphenol S (BPS) (Liao and Kannan, 2014). Because the BPA alternatives have a similar chemical structure and binding ability for the estrogen receptor (ER), they show toxicological effects (Nunez et al., 2001; Chen et al., 2002). BPB (2,2-bis(4-hydroxyphenyl)butane) is an analog of BPA and is mostly used for the manufacturing of phenolic resins (Cunha and Fernandes, 2010). It has been found at a concentration of 21.4% in food samples from Italian supermarkets (Cunha and Fernandes, 2010), 0.88%–11.94% in endometriosis of women, and 27.6% in the sera (Cobellis et al., 2009; Liao et al., 2012a). BPB was also found in indoor dust (Liao et al., 2012a, 2012c); however, there are very limited data on the human exposure of BPB. In a previous study, two of 20 tested human urine samples showed a positive result for BPB (Cunha and Fernandes, 2010). BPB has an estrogenic effect and is more resistant to biodegradation (Ike et al., 2006; Li et al., 2014); it causes a decrease in cortisol and corticosterone levels and can lead to DNA damage. Compared to BPA, BPB has much higher acute toxicity (Chen et al., 2002; Rosenmai et al., 2014). Another member of the BPA family, BPF (Bis(4-hydroxyphenyl)methane) has a lot of implications and is used in the manufacturing of polycarbonates and epoxy resins (Molina-Molina et al., 2013; Liao and Kannan, 2014). Several studies have shown the presence of BPF in the stuff food packages and in drinking water pumped through pipes made up of BPF (Cabado et al., 2008; Zou et al., 2012). BPF was also found in meat products, beverages, and vegetables (Gallart-Ayala et al., 2011; Liao and Kannan, 2013). BPF has been observed in different organs, including the reproductive organs, and can cross the placental barrier to reach the fetus (Cabaton et al., 2006).

Another replacement to BPA is BPS (bis(4-hydroxyphenyl)sulfone). It was first synthesized as a dye in 1869, and after the ban on BPA, it came into use in epoxy resins, infant feeding bottles, and thermal papers in 2006 (Liao et al., 2012b; Glausiusz, 2014). There have been several studies where BPS was detected in 81% of human matrices and 3% of breast milk and was found to result in DNA damage (Chen et al., 2002; Fic et al., 2013; Bergmann et al., 2015; Rochester and Bolden, 2015). The analogs of BPA such as BPB, BPF, and BPS have genotoxic effects and also induce oxidative stress. The endocrine disruptive nature of these analogs proposes that they are more harmful than BPA and are not safe alternatives for BPA (Feng et al., 2016). Bisphenol analogs have toxic effects including cytotoxicity, reproductive toxicity, neurotoxicity, and endocrine disruptive activity, reported by several studies. A study on BPA analogs shows that BPS and BPF have a similar potency for androgenic, antiandrogenic, estrogenic, and antiestrogenic activities (Liao et al., 2012c). In the present study, comparative toxicity effects of BPA and its analogs on the reproductive system of male rats were determined using both in vitro and in vivo approaches (Feng et al., 2016).

2. Material and methods

2.1. Chemicals and animals

BPA, BPB, BPF, and BPS (99% purity) were purchased from Santa Cruz Biotechnologies, USA. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and penicillin/streptomycin were all purchased from Thermo Fisher Scientific (Waltham, MA, USA). H_2O_2, Ca^{2+}, Mg^{2+}, Hank's balanced salt solution (HBSS), catalase (CAT), and N-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sprague–Dawley adult male rats (age: 80–90 days) were obtained from the animal facility of Quaid-i-Azam University, Islamabad. Animals were housed in cages made of steel, and each cage contained a maximum of five animals. Standard laboratory conditions were maintained before the start of the experiment. Room temperature was maintained at 22–25 °**C**, and a light/dark cycle was maintained. Animals were fed with laboratory feed, and tap water was available ad libitum for the animals. Protocols for handling of the animals were approved by the animal sciences department ethical committee.

2.2. Experimental design

Different experiments were designed to investigate the comparative effect of exposure to BPA and its analogs BPS, BPB, and BPF on the male reproductive system. In the in vitro experiment, the direct effect of BPA and its analogs (BPB, BPF, and BPS) on the concentration of testosterone and levels of antioxidant enzymes in the testes was checked, whereas in the in vivo experiment, the effects of different concentrations of BPA and its analogs on the reproductive system of male rats were +determined through a subchronic exposure study.

2.3. In vitro experiment

Sprague–Dawley male adults rats (n = 7) were used in this study. An in vitro experiment was performed to investigate the effect of the direct exposure of BPA and its analogs, namely, BPB, BPF, and BPS on the testosterone production and testicular antioxidant status. Different doses (0, 1, 10, and 100 ng/ml) of BPA and its analogs, namely, BPB, BPF, and BPS were used in this study. The doses of BPA and its analogs were selected in accordance with the exposure studies of (Hulak et al., 2013; Ullah et al., 2016). Stock solutions of bisphenols (BPA, BPB, BPF, and BPS) were prepared in ethanol. In vitro culturing of testicular slices was done according to (Ullah et al., 2016) with slight modifications. Testicular tissues were removed from the sacrificed animals and washed with saline. The dissected testes were cut into five equal parts and were processed in tubes. Dulbecco's media containing penicillin, sodium bicarbonate, and streptomycin were mixed with 0, 1, 10, and 100 ng/ml of BPA and its analogs BPB, BPS, and BPF, and the culture tubes were incubated for 2 h in a CO_2 incubator. After 2 h of incubation, the tissues were removed from the culture media and washed with saline. Ninety milligrams of the cultured tissue was homogenized in 3 ml of phosphate-buffered saline and centrifuged at 30,000 rpm for 30 min. The supernatant of the homogenate was collected and stored at −80 °C for hormonal assay and antioxidant assay.

2.4. In vivo experiment

Adult male Sprague–Dawley rats (70–80 days old; n = 91) were divided into 13 groups (n = 7 per group). Animals were exposed for 28 consecutive days orally to different concentrations (5, 50, and 500 mg/kg body weight/day) of BPA, BPB, BPF, and BPS. For a

subchronic exposure study, different doses of bisphenols were used. Ethanol was used for the preparation of stock solutions of bisphenols (BPA, BPB, BPF, and BPS); later, the stock solutions were diluted in saline, where the final concentration of ethanol was 0.1–0.5%. Animals were dissected out on the 29th day; testes were removed, and the blood was collected. Regarding the testicular tissues, the left testis and left epididymis were weighed and processed for biochemical analysis and the right testis and right epididymis were placed in 10% formalin for histology analysis. Blood collected from the animals was centrifuged at 3000 rpm for 10 min, and plasma was separated and stored at −20 °C until hormonal analysis.

2.5. Antioxidant enzymes

Tissues collected from both in vitro and in vivo studies were further processed for the antioxidant enzymes. Tissues were homogenized using an automatic homogenizer in phosphate-buffered saline and centrifuged at 30,000 rpm for 30 min. After centrifugation, the supernatant was removed and used for hormonal analysis, protein estimation, and antioxidant enzyme activities.

2.6. Catalase

CAT activity was determined by the method used by (Aebi, 1984), and the change in the absorbance due to H_2O_2 was measured in the tissues. In this assay, 50 ml of homogenate was diluted in 2 ml of phosphate buffer with pH of 7.0. After mixing it thoroughly, the absorbance was read at 240-nm wavelength with an interval of 15 s and 30 s. Change in the absorbance of 0.01 unit/min was defined as one unit of CAT.

2.7. Superoxide dismutase

Superoxide dismutase (SOD) activity was estimated by the method developed by (Kakkar et al., 1984). In this assay, the amount of chromogen formed was measured at 560-nm wavelength. The results were expressed in units/milligram of protein.

2.8. Peroxidase

Peroxidase (POD) activity in the homogenate was determined by the spectrophotometric method of (Carlberg and Mannervik, 1975). In this assay, 0.1 ml of homogenate was mixed with 0.1 ml of guaiacol, 0.3 ml of H_2O_2, and 2.5 ml of phosphate buffer, and the absorbance was read at 470-nm wavelength. Change in the absorbance of 0.01 unit/min was defined as one unit of POD.

2.9. Lipid peroxidation by TBARS

Activity of TBARS was determined in the homogenate by the method used by (Iqbal et al., 1996), and the results were expressed as TBARS/min/ml of plasma. In this assay, 0.1 ml of homogenate was mixed with 0.29 ml of phosphate buffer, 0.1 ml of trichloroacetic acid, and 1 ml of trichlorobarbituric acid followed by heating at 95 °**C** for 20 min and then shifting to an ice bath before centrifuging at 2500 for 10 min. The samples were read using a spectrophotometer at 535-nm wavelength.

2.10. Total protein content

AMEDA Laboratory diagnostic kit was used for the determination of total protein in the tissue. The results of total protein were calculated by plotting absorbance of the standard against that of samples. These values were expressed as mg/g of tissue.

2.11. Reactive oxygen species

The assay of reactive oxygen species (ROS) was performed according to the method of (Hayashi et al., 2007), and for the presentation of mean values, the assay was repeated multiple times. In this assay, 5 ml each of H_2O_2 standards and the homogenate was mixed with 140 ml of sodium acetate buffer with pH 4.8 in a 96-well plate, and this plate was incubated at 37 °C for 5 min. After incubation, 100 ml of DEPPD and ferrous sulfate mix sample was added in each well at a ratio of 1:25, and the plate was incubated again at 37 °C for 1 min. With an interval of 15 s for 3 min, the absorbance was read at 505-nm wavelength using a microplate reader.

2.12. Hormonal analysis

For the quantitative measurement of testosterone concentrations in the tissues, an EIA kit was used, and the assay was performed according to the instructions in the kit.

2.13. Tissue histology

Testicular tissues (testes and epididymis) were fixed in formalin for 48 h. The tissues were dehydrated with different grades of alcohol and cleared with xylene. The paraffin sections (5 μm) were cut and stained with hematoxylin and eosin to assess standard histology and morphometry. Testicular sections from 10 to 20 rats per group were digitized under Leica Microscope (New York Microscope Company) equipped with a digital camera (Canon, Japan).

For morphometry analysis, the images were taken at 20x and 40x magnifications, and the results were processed with Image J software. The area of different sections was calculated with the method of (Jensen, 2013). From 20x images, 30 pictures per animal were selected, and known area of different areas of the intestinal space, epididymis tubules, and seminiferous tubules was measured using the software program. The number of different cell types (spermatids, spermatogonia, and spermatocytes) and area was calculated, and comparison of different groups with the control was made.

2.14. Statistical analysis

The Dunnett's multiple comparison test, which followed analysis of variance (ANOVA), was used for the comparison of different groups with the control using GraphPad Prism software. Values were expressed as mean ± SEM and were considered significant at $P < 0.05$.

3. Results

3.1. In vitro effect of the bisphenol analogs BPB, BPF, and BPS on testicular antioxidant enzymes, ROS, and testosterone secretion in the rat testes

In the testicular tissue, antioxidant enzymes (CAT, POD, and SOD), ROS, and lipid peroxidation (LPO) were determined after incubation with different concentrations of bisphenols, namely, BPA, BPB, BPF, and BPS, for 2 h. There was no significant difference observed in CAT activity in any of the treated group as compared to the control group. Similarly, there was also no significant difference observed in the values of POD of all treated groups as compared to the control group (Table 1). SOD values were also not very different from the activity of both CAT and POD (Table 1).

ROS and LPO, which are considered as oxidative stress markers, were observed in the in vitro treated groups of bisphenols (BPA,

Table 1
In vitro Effect of Bisphenol A analogs BPB, BPF and BPS on antioxidants and testosterone in rat testis. Values are expressed as mean ± SEM.

Treatments	Parameters					
	CAT (u/mg Protein)	POD (nmole)	SOD (u/mg protein)	LPO (min/mg Tissue)	Total ROS (U/g tissue)	Testosterone (ng/g tissue)
Control	8.12 ± 0.6	9.92 ± 2.7	10.51 ± 2.9	29.10 ± 0.2	25.8 ± 0.85	54.27 ± 0.4
BPA 1 ng/ml	7.18 ± 0.5	4.42 ± 0.6	7.18 ± 0.9	16.17 ± 1.5	33.6 ± 3.4	50.77 ± 4.7
BPA 10 ng/ml	3.94 ± 0.4	6.92 ± 1.1	13.27 ± 1.9	36.06 ± 2.8	27.6 ± 2.2	45.07 ± 2.0
BPA 100 ng/ml	6.54 ± 0.9	6.45 ± 1.3	13.40 ± 2.6	41.21 ± 4.8	34.6 ± 3.9	41.90 ± 0.2
BPB 1 ng/ml	2.79 ± 0.4	8.26 ± 2.5	11.75 ± 1.8	40.97 ± 5.3	33 .0 ± 3.3	42.59 ± 0.1
BPB 10 ng/ml	4.39 ± 0.7	9.86 ± 2.9	6.51 ± 1.2	40.91 ± 2.4	37.4 ± 2.5*	40.37 ± 3.1
BPB 100 ng/ml	6.90 ± 0.8	5.82 ± 0.8	14.25 ± 4.2	41.99 ± 4.5	36.8 ± 2.7	42.15 ± 3.7
BPF 1 ng/ml	4.64 ± 1.3	3.50 ± 0.6	13.96 ± 3.5	36.61 ± 7.0	34.8 ± 0.7	42.79 ± 0.4
BPF 10 ng/ml	6.63 ± 1.0	5.41 ± 1.3	11.00 ± 2.9	42.86 ± 5.3	43.8 ± 0.7***	42.37 ± 0.3
BPF 100 ng/ml	5.16 ± 3.9	12.12 ± 4.2	10.49 ± 3.6	38.06 ± 6.1	41.2 ± 4.1**	41.46 ± 0.9
BPS 1 ng/ml	3.69 ± 1.5	17.55 ± 14.7	13.11 ± 1.7	37.05 ± 1.8	26.4 ± 2.9	53.15 ± 1.3
BPS 10 ng/ml	6.92 ± 3.9	11.73 ± 4.6	16.25 ± 0.8	42.17 ± 1.1	23.0 ± 0.7	51.65 ± 5.7
BPS 100 ng/ml	3.81 ± 0.8	7.24 ± 2.8	12.39 ± 0.8	52.49 ± 1.0*	39.8 ± 4.2**	52.00 ± 1.7

Values are expressed as mean ± SEM.
*, **, *** indicate significant difference at probability value P < 0.05, P < 0.01 and P < 0.001 compared to control.
ANOVA followed by Dunnet's Comparison test.

BPB, BPF, and BPS), and the results are presented in Table 1. Regarding the LPO levels, a significant increase was observed in the BPS 100 ng/ml group (P < 0.05) when compared to the control group. However, other doses did not increase LPO levels as compared to the control group. ROS levels significantly decreased with an increasing dose of bisphenol in the treated groups as compared to the control group. A significant increase was observed in the BPB 10 ng/ml (P < 0.05) and BPF 10 ng/ml (P < 0.01) groups when compared to the control groups. ROS values were significantly increased (P < 0.01) in the BPF 100 ng/ml and BPS 100 ng/ml treated groups. However, the other treated groups did not have increased ROS values as compared to the control group.

Testosterone levels decreased after treatment of testis with 2 h of incubation with BPA and its analogs, namely, BPB, BPF, and BPS. All the doses of BPA and its analogs reduced testosterone levels, but a significant reduction was not observed in the treated groups when compared to the control group (Table 1).

3.2. Effect of BPA and its analogs BPB, BPF, and BPS on body weight gain and testicular weight after subchronic administration

Body weight gain after 28 days of exposure showed no significant change in all the treated groups as compared to the control group. Similarly, in the left and right testes of all the treated groups, no significant change was observed when compared to the control group (Table 2).

3.3. Biochemical parameters of the rat testes after subchronic treatment with BPA and its analogs BPB, BPF, and BPS

Details of Antioxidant enzymes, SOD, and POD in the testicular tissue after 28 days of subchronic exposure are presented in Table 2. No significant change was observed in SOD activity in the treated groups compared to the control group. However, for POD activity, a significant reduction was observed in the BPA 50 mg/kg group (P < 0.001) when compared to the control group. POD activity was reduced significantly (P < 0.01, P < 0.01, and P < 0.05) in BPB 5, 25, and 50 mg/kg treated groups. Similarly, BPF treatment caused significant reduction (P < 0.01, P < 0.05, and P < 0.01) at dose levels of 5, 25, and 50 mg/kg. On the other hand, PBS 5 mg/kg significantly reduced (P < 0.05) POD in the testicular tissues; however, the other doses of BPS did not reduce POD level as compared to the control.

Activity of CAT in the testicular tissues after 28 days of exposure showed a significant reduction in the BPA 5 and 25 mg/kg groups (P < 0.05 and P < 0.01) as compared to the control group. BPB at a dose of 25 mg/kg caused a significant reduction (P < 0.05) in CAT activity in testicular tissues when compared to the control group. Similarly, BPF at doses 25 and 50 mg/kg reduced (P < 0.01) CAT

Table 2
Bisphenol A and (BPB, BPF and BPS) sub chronic effect on the different parameters of rat testis.

Treatments	Parameters				
	Body weight gain (g)	Right Testis weight (g)	Left testis weight (g)	SOD (u/mg protein)	POD (nmole)
Control	33 ± 4.11	1.06 ± 0.05	1.13 ± 0.02	48.54 ± 1.51	15.15 ± 0.20
BPA 5 mg/kg	25 ± 3.81	1.16 ± 0.73	1.16 ± 0.08	26.29 ± 5.71	14.77 ± 0.68
BPA 25 mg/kg	22 ± 3.21	1.02 ± 0.07	1.02 ± 0.04	20.38 ± 4.38	13.30 ± 0.96
BPA 50 mg/kg	22 ± 4.10	1.11 ± 0.08	1.04 ± 0.05	38.26 ± 8.20	11.95 ± 0.22***
BPB 5 mg/kg	29 ± 3.22	1.21 ± 0.05	1.16 ± 0.05	25.96 ± 11.42	12.55 ± 0.49**
BPB 25 mg/kg	23 ± 3.81	1.12 ± 0.05	1.12 ± 0.06	32.54 ± 3.38	13.11 ± 0.59*
BPB 50 mg/kg	26 ± 4.01	1.12 ± 0.06	1.16 ± 0.05	29.81 ± 8.12	12.81 ± 0.28*
BPF 5 mg/kg	27 ± 2.71	1.01 ± 0.06	1.04 ± 0.07	21.32 ± 3.87	12.55 ± 0.43**
BPF 25 mg/kg	23 ± 2.23	1.12 ± 0.04	1.14 ± 0.09	33.34 ± 7.42	12.85 ± 0.09*
BPF 50 mg/kg	27 ± 3.23	0.97 ± 0.11	1.26 ± 0.05	36.02 ± 10.65	12.61 ± 0.39**
BPS 5 mg	25 ± 4.10	1.18 ± 0.04	1.12 ± 0.04	30.59 ± 7.15	12.75 ± 0.59*
BPS 25 mg/kg	26 ± 3.23	1.01 ± 0.07	0.97 ± 0.03	39.63 ± 8.17	13.77 ± 0.25
BPS 50 mg/kg	28 ± 3.81	1.01 ± 0.2	1.00 ± 0.09	28.57 ± 6.48	13.34 ± 0.44

Values are expressed as mean ± SEM.
*, **, *** indicate significant difference at probability value P < 0.05, P < 0.01 and P < 0.001 compared to control.
ANOVA followed by Dunnet's Comparison test.

activity as compared to the control group. On the other hand, BPS at a dose of 5 and 50 mg/kg significantly reduced ($P < 0.01$ and $P < 0.05$) CAT activity in testicular tissues. No significant difference was observed in the other treated groups.

LPO, a well-known oxidative stress marker, was determined, and the result is presented in Table 3. A significant increase in the LPO (T-BARS) content was observed in the BPA 50 mg/kg group ($P < 0.05$) when compared to the control group. LPO content reduced significantly ($P < 0.01$) in the BPB 50 mg/kg treated group. Similarly, BPF treatment caused a significant reduction ($P < 0.001$) at a dose level of 50 mg/kg; however, BPF at doses of 5 and 25 mg/kg did not affect POD activity. BPS at a dose of 50 mg/kg significantly reduced ($P < 0.01$) LPO activity in the testicular tissues. However, the other doses of BPS did not show a significant effect as compared to the control group.

Results of the total ROS level in the different treatment groups and the control group is presented in Table 3. A significant increase was observed in the BPA 50 mg/kg group ($P < 0.001$) when compared to the control group. The total ROS level was significantly increased ($P < 0.001$) in the BPB 50 mg/kg group when compared to the control group. Similarly, BPF and BPS treatments caused a significant increase ($P < 0.01$ and $P < 0.001$, respectively) at a dose level of 50 mg/kg. However, the total ROS level was not altered by BPS at doses of 5 and 25 mg/kg.

Total protein content in the testes after 28 days of exposure showed a significant reduction in the BPA 5 mg/kg ($P < 0.05$), BPA 25 mg/kg ($P < 0.01$), and BPA 50 mg/kg ($P < 0.05$) groups as compared to the control group. Protein concentration was significantly reduced ($P < 0.05$) in the BPB 5 and 50 mg/kg treated groups. On the other hand, the BPF 5 and 25 mg/kg treatment groups showed a significant reduction ($P < 0.05$ and $P < 0.001$, respectively) in protein levels as compared to the control group. Similarly, BPS at doses 5, 25, and 50 mg/kg reduced total protein content as compared to control (Table 3).

3.4. Effect of BPA and its analogs BPB, BPF, and BPS on the intratesticular and plasma testosterone levels in rats

Plasma testosterone concentrations in different treatment groups and control are presented in Table 4. A significant reduction was observed in the BPA 5 mg/kg ($P < 0.05$), BPA 25 mg/kg ($P < 0.01$), and BPA 50 mg/kg ($P < 0.05$) groups when compared to the control group. Testosterone concentration was significantly reduced ($P < 0.05$ and $P < 0.01$, respectively) in the BPS 5 and 50 mg/kg treated groups. Similarly, BPF treatment caused a significant reduction ($P < 0.05$) at dose levels of 5 and 50 mg/kg. However, testosterone concentrations did not show a significant difference in the BPF 25 mg/kg treated group. On the other hand, BPS at a dose of 50 mg/kg significantly reduced ($P < 0.05$) testosterone concentration in the plasma; however, other doses did not reduce plasma testosterone concentration as compared to the control group.

Intratesticular testosterone concentration in the testis after 28 days of exposure showed a significant reduction in the BPA 25 and 50 mg/kg groups ($P < 0.05$, $P < 0.01$) as compared to the control group. All doses of BPB and BPF caused a significant reduction ($P < 0.01$) in intratesticular testosterone concentration when compared to the control group. Similarly, BPS at doses 5 and 50 mg/kg reduced ($P < 0.05$ and $P < 0.01$, respectively) intratesticular testosterone concentration as compared to the control group. Intratesticular testosterone concentration was not different in the BPA 5 mg/kg and BPS 25 mg/kg groups compared to that in the control group.

3.5. Testicular and epididymis morphological changes after exposure to BPA and its analogs BPB, BPF, and BPS

Details on testis and epididymis morphological changes in the area of seminiferous tubule and interstitium, seminiferous tubule diameter, and epithelial height after 28 days of exposure are presented in Table 5 and Fig. 1. There was no significant difference observed in the area of seminiferous tubule % and area of interstitium % of different treatment groups as compared to the control group. Similarly, a significant difference was not observed in the diameter of seminiferous tubule in all treated groups as compared to the control group. On the other hand, BPA at a dose of 50 mg/kg significantly reduced ($P < 0.05$) epithelial height. A significant reduction ($P < 0.01$) in the epithelial height was also observed in the BPB and BPF 50 mg/kg groups as compared to the control group. Similarly, BPS at a dose of 50 mg/kg reduced ($P < 0.05$) epithelial height as compared to the control group. Epithelial height was not different in the BPA, BPB, BPF, and BPS 5 and 25 mg/kg groups compared to the control group.

In the control group, testis with thick epithelium, sperm-filled lumen, and seminiferous tubules were observed, and the details are shown in Fig. 1. The arrangement and shape of the seminiferous tubules were not very different in all treated groups when compared to the control group. However, the pattern of epithelium

Table 3
Effect of sub chronic bisphenols (A, B F and S) exposure on the biochemical parameters of male rats.

Treatments	Parameters			
	CAT (u/mg Protein)	LPO (nM TBARS/min/mg protein)	Total ROS (U/g tissue)	Protein (mg/0.5 g)
Control	14.87 ± 0.27	13.92 ± 0.24	0.74 ± 0.01	333.91 ± 09.28
BPA 5 mg/kg	13.11 ± 0.44*	12.84 ± 0.40	0.90 ± 0.05	283.89 ± 23.59*
BPA 25 mg/kg	12.63 ± 0.38**	13.35 ± 0.32	0.77 ± 0.01	270.23 ± 08.79**
BPA 50 mg/kg	13.58 ± 0.40	15.53 ± 0.24*	1.30 ± 0.04***	280.90 ± 11.92*
BPB 5 mg/kg	14.11 ± 0.28	14.15 ± 0.51	0.95 ± 0.03	284.77 ± 04.02*
BPB 25 mg/kg	13.18 ± 0.23*	14.01 ± 0.38	0.86 ± 0.10	291.80 ± 18.68
BPB 50 mg/kg	13.89 ± 0.23	15.78 ± 0.27**	1.38 ± 0.07***	285.08 ± 04.61*
BPF 5 mg/kg	13.53 ± 0.43	14.40 ± 0.37	0.74 ± 0.07	281.28 ± 16.76*
BPF 25 mg/kg	12.86 ± 0.34**	14.64 ± 0.24	0.79 ± 0.03	264.26 ± 04.89***
BPF 50 mg/kg	12.57 ± 0.34**	15.99 ± 0.22***	1.11 ± 0.13**	288.07 ± 03.03
BPS 5 mg	12.68 ± 0.52**	14.22 ± 0.31	0.82 ± 0.04	283.61 ± 05.44*
BPS 25 mg/kg	13.66 ± 0.44	14.22 ± 0.31	0.75 ± 0.06	284.78 ± 04.83*
BPS 50 mg/kg	13.16 ± 0.58*	15.82 ± 0.24**	1.18 ± 0.08***	273.02 ± 08.89**

Values are expressed as mean ± SEM.
*, **, *** indicate significant difference at probability value $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control.
ANOVA followed by Dunnet's Comparison test.

Table 4
Sub chronic effect of Bisphenols (A, B, F and S) on the plasma and intra-testicular testosterone hormone production in rats.

Treatments	Parameters	
	Plasm Testosterone (ng/ml)	Intra-Testicular Testosterone (ng/g tissue)
Control	5.90 ± 0.18	54.27 ± 0.82
BPA 5 mg/kg	3.96 ± 0.23*	50.77 ± 2.74
BPA 25 mg/kg	3.77 ± 0.29**	45.07 ± 1.59*
BPA 50 mg/kg	3.88 ± 0.41*	41.90 ± 0.30**
BPB 5 mg/kg	3.81 ± 0.37*	42.59 ± 0.78**
BPB 25 mg/kg	4.16 ± 0.26*	44.03 ± 0.33**
BPB 50 mg/kg	3.71 ± 0.22**	42.15 ± 3.12**
BPF 5 mg/kg	3.89 ± 0.17*	43.46 ± 0.81**
BPF 25 mg/kg	4.29 ± 0.36	43.70 ± 0.55**
BPF 50 mg/kg	3.99 ± 0.14*	42.46 ± 2.26**
BPS 5 mg	4.39 ± 0.54	45.82 ± 0.68*
BPS 25 mg/kg	4.45 ± 0.31	47.65 ± 0.94
BPS 50 mg/kg	3.93 ± 0.30*	44.71 ± 0.17**

Values are expressed as mean ± SEM.
*, **, *** indicate significant difference at probability value $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to control.
ANOVA followed by Dunnet's Comparison test.

Table 5
Oral Sub Chronically administered rats with Bisphenol A and its analogs (B, F and S) testis morphometry.

Treatments	Parameters			
	Area of seminiferous tubule (%)	Area of Intrstitium (%)	Seminiferous tubule diameter (μm)	Epithelial height
Control	85.64 ± 1.89	15.87 ± 1.15	207.62 ± 1.79	71.48 ± 1.92
BPA 5 mg/kg	83.83 ± 1.31	16.06 ± 1.47	201.30 ± 3.16	69.05 ± 1.03
BPA 25 mg/kg	82.96 ± 1.15	16.56 ± 1.21	205.38 ± 1.57	65.55 ± 1.30
BPA 50 mg/kg	81.70 ± 1.64	17.28 ± 1.33	204.06 ± 1.50	59.38 ± 2.20*
BPB 5 mg/kg	83.92 ± 1.61	16.97 ± 1.22	205.48 ± 1.62	68.89 ± 1.30
BPB 25 mg/kg	82.66 ± 1.22	16.50 ± 1.48	205.68 ± 1.48	69.21 ± 1.32
BPB 50 mg/kg	83.78 ± 1.25	16.80 ± 1.39	204.10 ± 1.27	58.04 ± 2.75**
BPF 5 mg/kg	84.53 ± 1.39	16.62 ± 1.57	204.44 ± 1.61	69.22 ± 2.13
BPF 25 mg/kg	83.64 ± 1.46	16.69 ± 1.46	202.53 ± 1.74	66.52 ± 1.77
BPF 50 mg/kg	83.79 ± 1.36	17.40 ± 1.42	204.28 ± 1.23	59.20 ± 2.54**
BPS 5 mg	84.50 ± 1.31	17.70 ± 1.47	203.84 ± 1.26	69.03 ± 2.68
BPS 25 mg/kg	83.68 ± 1.44	17.68 ± 1.51	204.48 ± 1.59	64.22 ± 1.89
BPS 50 mg/kg	84.36 ± 1.34	16.57 ± 1.75	203.66 ± 1.46	58.64 ± 2.75*

Values are expressed as mean ± SEM.
*, ** indicate significant difference at probability value $P < 0.05$ and $P < 0.01$ compared to control.
ANOVA followed by Dunnet's Comparison test.

was thin, and the number of secondary spermatocytes was reduced in the treated groups when compared to the control group. The groups with a higher dose (50 mg/kg/day) were observed to have very few tubules, and there were no elongated spermatids in the lumen of the treated group when compared to those in the control group (Fig. 1).

Morphometry of different parameters of epididymal caput and cauda regions after 28 days of subchronic exposure did not show any significant difference in any of the parameters (tubular and lumen diameter, epithelial height, and percentage of epithelium and lumen) as compared to the control group.

4. Discussion

Restriction on the use of BPA in the market has led to the use of its alternatives, namely, BPB, BPF, and BPS, which are reported to be unsafe. It is thought that the production of these analogs is going to increase in the future owing to the ban on BPA use in several countries of the world. It is not only concerning but also alarming that these analogs have been reported in several edible samples, which increase the threat of both general and occupationally exposed people. Owing to the similarity in its structure with BPA, these analogs can act like endocrine disrupters (Usman and Ahmad, 2016). In vitro data provided in mammals suggest that both BPF and BPS are capable of binding many receptors and change testosterone secretions, found by fetal testis assay, and can induce cell proliferation (Kitamura et al., 2005; Delfosse et al., 2012; Molina-Molina et al., 2013; Eladak et al., 2015). Thus far, there are very little data available using in vivo studies in mammalian and nonmammalian models, but some studies have shown that these compounds have impact on the expression of hormone-regulated genes and can exhibit reproductive and developmental effects (Ji et al., 2013; Naderi et al., 2014; Kinch et al., 2015; Cano-Nicolau et al., 2016). The present data are raising concerns that whether the so-called exposure to safer alternatives to BPA is either safe or more threatening to living organisms. Very limited data are available on the analogs of BPA, and these can confirm whether they are really safe or it is just a commercial shift to continue the BPA family in the market. We conducted two experiments both in vivo and in vitro to show the toxic effect of BPA and its analogs on testicular tissues and reproductive function in male rats; based on the current literature, BPS, BPF, and BPB have already been detected in consumer products as alternatives for BPA (Rochester and Bolden, 2015).

In the in vitro study, we aimed to assess the effects of BPA and its commonly used analogs BPB, BPF, and BPS, on the antioxidant status of testicular tissues. By incubating testicular tissues for 2 h with BPA and its analogs (BPB, BPF, and BPS), no significant change in the activity of CAT, SOD, and POD was observed. Antioxidant

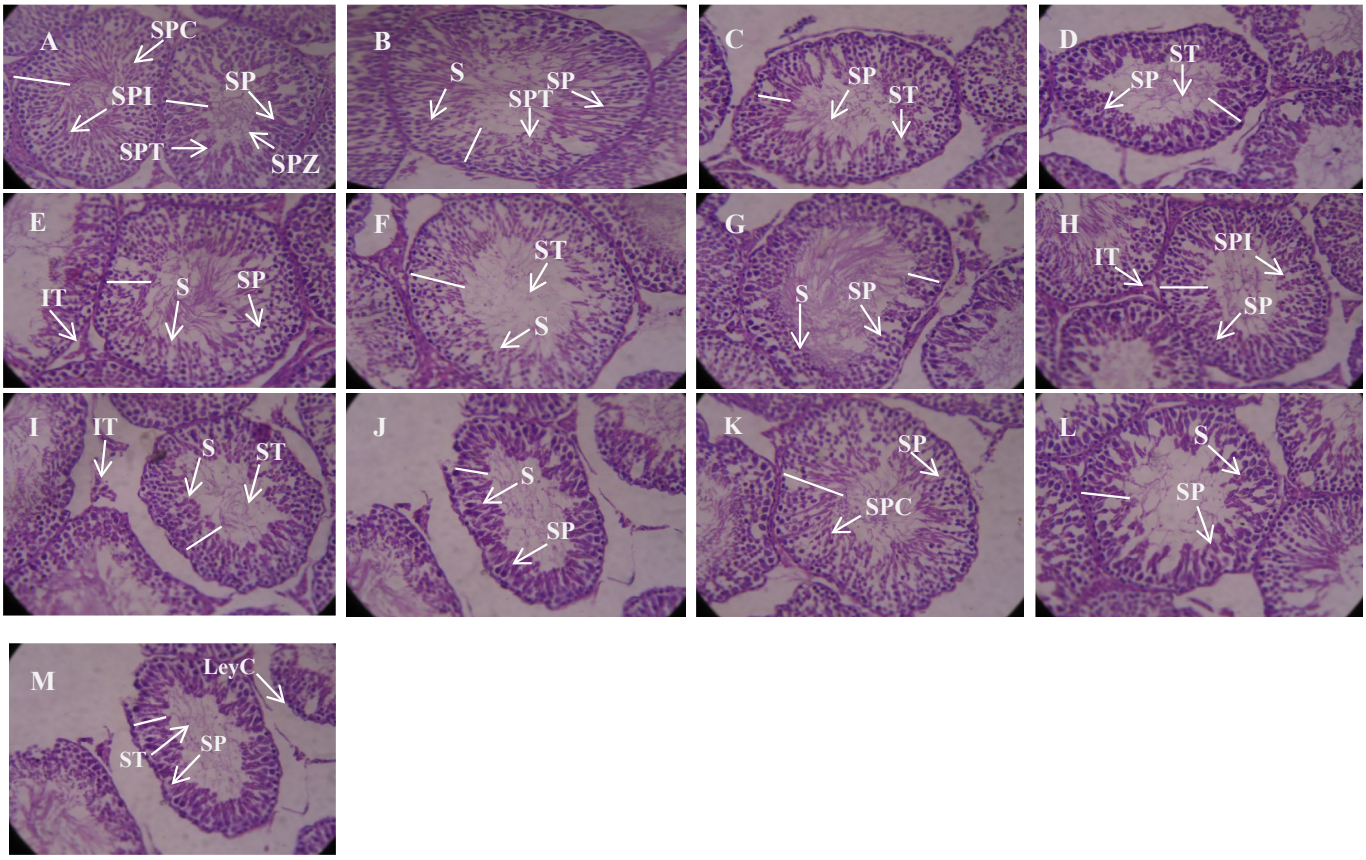

Fig. 1. Photomicrographs of rats testicular tissues of control and treated animals with different concentrations of BPA, BPB, BPF and BPS. The control (A) reveals normal germ cells: Spermatogonia (SP), spermatocytes (SPC), Spermatids (SPT), spermatozoa (SPZ). (B, C and D) treated groups with BPA (5, 25 and 50 mg/kg/day) showing change in the testicular tissues seminiferous tubules with epithelium (Line without arrow head) and spermatids (White arrow): (E, F and G) BPB (5, 25 and 50 mg/kg/day) showing change in the testicular parenchyma, abundant interstitial tissues (IT), absence of sperm in lumen and different cells (White arrow): (H, I and J) BPF (5, 25 and 50 mg/kg/day) treated groups presenting irregular seminiferous tubules with disturbed epithelium (Line without arrow head) and elongated spermatids (White arrow): (K, L and M) BPS (5, 25 and 50 mg/kg/day) treated groups presenting seminiferous tubules with germ cells, leydig cells (LeyC) and absence of sperm in lumen of tubules. ST: seminiferous tubules; SP: spermatogonia; SPC: spermatocytes; SPT: spermatids; SPZ: spermatozoa; IT: interstitial tissue; LeyC: Leydig cell (White arrow). H&E (x40).

enzymes such as CAT and SOD play a vital role in the mechanism against oxidative stress in the body (Pandey and Rizvi, 2010). ROS formation is adversely affected by many toxicants that damage cellular network and structure. The toxic influence of phenols is associated with ROS, suggested by many researchers such as (Michałowicz and Duda, 2007) who showed that BPA plays an important role in the formation of ROS and oxidation of many cellular biomolecules in the body (Zhan et al., 2006; Korkmaz et al., 2010). However, the values of ROS and POD in our study showed significant difference as compared to the control group, which and these values are similar to the in vitro effect of BPS observed by (Ullah et al., 2016). BPA and its analogs have also been observed to increase the levels of ROS in human peripheral blood mononuclear cells (Michałowicz, 2014). A study by (Maćczak et al., 2017) showed that the mechanism of oxidative action of BPA and its analogs BPB, PBF, and BPAF increased the level of ROS, caused LPO, and also altered the activities of SOD and CAT in mature erythrocytes. In the in vivo study, we observed that there was a dose-dependent effect of bisphenols (A, B, F, and S) on the oxidative stress in the reproductive system of rats. In the groups treated at higher doses, we observed that there was a significant change in the histology of the reproductive tissues by reducing the number of sperms in the lumen of the epididymis and decreasing the height of epithelial tissues of seminiferous tubules. This is not surprising as estrogen, while essential for normal epididymis function, has inhibitory effects on the brain, pituitary, and gonadal axis in males, and it is well documented that elevated E2 inhibits spermatogenesis and testicular testosterone secretion (Richter et al., 2007). Interference with androgen action during gonadal development can also cause abnormalities of the male reproductive system (Lee et al., 2003). High doses also induced higher oxidative stress in the tissues than the low doses and control dose. The above changes can be due to the increase in ROS (Devasagayam et al., 2004). ROS, which is produced in the mitochondria, produces free oxygen ions during normal metabolism, and these ions help in homeostasis and cell signaling (Rejitha and Karthiayini, 2013). If this level of ROS continues at the same rate, it will result in DNA damage and damage to lipids and proteins. To overcome this situation, the cell activates its antioxidant enzyme production, which is the self-defense mechanism of the body and helps in reducing the levels of ROS (Kaul and Forman, 2000). When cells are unable to detoxify ROS, they go into oxidative stress, which causes a reduction in the level of antioxidant enzymes (Kaul and Forman, 2000; Pérez et al., 2009).

From the in vitro study results, it seems that bisphenol levels caused the induction of ROS, which leads to a surge in LPO levels and activation of antioxidant enzymes of the tissues, and this fact is in line with the earlier study where in the in vitro study, the degradation of proteins in cells occurred because of bisphenol exposure (Michałowicz et al., 2015). In some of the other studies, it was also found that if this oxidative stress persists, it can cause injury to the cell membranes, known as LPO (Feng et al., 2012; Lee et al., 2013; Mokra et al., 2015). BPA and BPS also caused protein and

DNA damage in cells in the in vitro studies (Rotroff et al., 2013). Oxidative stress was also observed in the in vivo studies where the levels of ROS increased and LPO levels also increased to an observable level. There was also a change observed in the SOD and CAT activities of different treated groups, which also indicates oxidative stress in the tissue. These high levels of ROS and LPO also indicate that this change occurred because of the oxidative stress caused by bisphenols (A, B, F, and S), which reduced the level of antioxidant enzymes and proteins in the tissues, similar to the findings of a prior study by (Radák et al., 1999).

Testosterone concentrations in the in vitro study showed no significant change when matched to the control group; however, there was a substantial change noted in both intratesticular and plasma testosterone concentrations in the treated groups of an in vivo study as compared to the control group. Both intratesticular and plasma testosterone concentrations reduced in the treated groups compared to those in the control group. On the other hand, in the in vitro testicular tissues, we observed less change in the testosterone production, which can be due to the short incubation period. (Rosenmai et al., 2014) also investigated the effects of the BPA alternatives BPF and BPS on steroidogenesis where they observed that BPA and its analogs BPF and BPS altered the steroidogenesis pathway by increasing and decreasing the concentrations of different hormones and showing the tendencies same as those we found in our study (García et al., 2012; Rosenmai et al., 2014).

Reproductive hormones and cellular interactions in the testes control the process of spermatogenesis. A disturbance in the antioxidant enzymes due to ROS leads into altered spermatogenesis. In the present study, the higher ROS levels have altered the levels of androgens. These altered levels of androgens lead to less number of spermatids, thin epithelial height, and seminiferous tubules in the testicular tissues and reduced concentrations of testosterone in the control group when compared to the treated groups. In the earlier studies, it was observed that exposure to BPA and BPS alters steroidogenesis and reduces gene transcripts for gonadotropin-releasing hormone (GnRH) and oxidative stress in different tissues (Ji et al., 2013; Allard, 2014; Manfo et al., 2014; Ullah et al., 2016; Jambor et al., 2017; Lee et al., 2018). Prominently, the present study shows that bisphenols (A, B, F, and S) act as inducers for the oxidative stress, which alters spermatogenesis in the testis by reducing the testosterone secretion. In this context, studies based on both in vivo and in vitro specific mechanisms are needed to determine the GnRh transcripts, which may show the cell- and tissue-specific response in the environment hazard assessment of these substitutes of bisphenols and EDCs; this will also highlight the molecular mechanism in understanding the comparison of in vitro and in vivo studies.

5. Conclusions

In the present study, findings of both in vitro and in vivo studies suggest that BPA and its analogs, namely, BPB, BPF, and BPS not only show antiandrogenic properties but also lead to oxidative stress, which cause disturbances in the reproductive function of adults. However, to understand the exact mechanism of these conditions, different studies need to be carried out both in vivo and in vitro with different low and high doses of all these analogs of BPA to know the biochemical, physiological, and endocrine effects in different animals.

Declaration of interest

The authors report no declarations of interest.

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Impact of low-dose chronic exposure to bisphenol A and its analogue bisphenol B, bisphenol F and bisphenol S on hypothalamo-pituitary-testicular activities in adult rats: A focus on the possible hormonal mode of action

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ABSTRACT

Bisphenol A an estrogen-mimic endocrine disrupting chemical, used to manufacture polycarbonate plastics and epoxy resins with toxic effects for male reproduction. Due to its toxicity, industries have started to replace it with other bisphenols. In this study, the toxicity of BPA analogues (BPB, BPF and BPS) was evaluated in a chronic study. We investigated whether the chronic exposure to low bisphenols doses affects spermatogenesis with outcomes on oxidative stress and male reproductive system. Male rats (22 day old) were exposed to water containing 0.1% ethanol for control or different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 μg/L) in drinking water for 48 weeks. Results of the present study showed a significant alteration in the gonadosomatic index (GSI) and relative reproductive organs weights. Oxidative stress in the testis was significantly elevated while sperm motility, Daily sperm production (DSP) and number of sperm in epididymis were reduced. Plasma testosterone, LH and FSH concentrations were reduced and estradiol levels were high in 50 μg/L exposed group. These results suggest that exposure to BPA and its analogues for chronic duration can induce structural changes in testicular tissue and endocrine alterations in the male reproductive system.

1. Introduction

Plasticizer such as bisphenol A (BPA) is an environmental pollutant detected in wildlife, humans samples and environment (Corrales et al., 2015). BPA exposure is associated with many human diseases and is suspected to affect many body's physiological functions (Chen et al., 2016a; Chevalier and Fénichel, 2015; Seachrist et al., 2016). Having several concerns for a safer world of BPA there have been several alternatives of BPA introduced into environment known as BPA analogues (Chen et al., 2016a). Bisphenol B (BPB), bisphenol F (BPF) and bisphenol S (BPS) are BPA alternatives which are used for the production of Plastics, epoxy resins, polycarbonates for lining large food containers, water pipes and coatings of Food containers, dyes, paper products and food packaging materials (Chen et al., 2016a; Danzl et al., 2009; Eladak et al., 2015; Goodson et al., 2002; Kinch et al., 2015; Rochester and Bolden, 2015; Yang et al., 2014). BPA analogues have increased concerns regarding emerging environmental pollutants where some of these analogues are detected in concentrations higher than BPA (Caballero-Casero et al., 2016; Chen et al., 2016a). For example, in a study from Italy the concentrations of BPB were higher than BPA in serum samples of healthy women and endometriotic women (Caballero-Casero et al., 2016). Similarly, in another study from Saudi Arabia in the urine of general population the concentrations of both BPS and BPF were higher than BPA (Chen et al., 2016a). In another study food products sold in New York and Albany were analyzed and 75% were detected with bisphenols measurable amounts (Liao and Kannan, 2013). BPS and BPF have been identified up to detectable amounts in food items and paper products (Goldinger et al., 2015; Liao and Kannan, 2014b; Russo et al., 2017). Across the Globe several studies have shown detectable amounts of BPA analogues in the urinary samples, umbilical cord samples and maternal samples (Asimakopoulos et al., 2016; Heffernan et al., 2016; Liu et al., 2017; Lu et al., 2016; Ye

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et al., 2015). BPA and its analogues observed in in vitro studies induced a number of physiological changes in cell lines of red blood cells, pre-adipocytes and testis (Boucher et al., 2016; Desdoits-Lethimonier et al., 2017; Maćczak et al., 2017; Mokra et al., 2017). Studies on rodents show that BPA analogues affects hormone concentrations, testis function, sperm production and sperm DNA damage (Castro et al., 2013; Li et al., 2016; Oliveira et al., 2017; Shi et al., 2017). Many studies of bisphenol A analogues suggest that these chemicals have greater neuroendocrine disruptive effects as BPA where they lead to complex behavioral changes in rodent species (Catanese and Vandenberg, 2016; Kim et al., 2015; Ohtani et al., 2017; Rosenfeld, 2017). Where, these chemicals also affect the gene expression in hypothalamus and other brain areas (Cano-Nicolau et al., 2016; Huang et al., 2016; Qiu et al., 2015, 2018; Zhang et al., 2017, 2018). BPA analogues have also been studied to induce hormonal imbalance in E2 synthesis, thyroid hormone production and testosterone levels (Cano-Nicolau et al., 2016; Kwon et al., 2016; Le Fol et al., 2017; Li et al., 2016).

In vitro and in vivo studies regarding BPA analogues are scare and limited data have shown that these chemicals have reproductive toxicity (Chen et al., 2016a; Naderi et al., 2014). These chemicals also have endocrine disrupting actions in vivo studies and are also estrogenic in nature (Kitamura et al., 2005; Rosenmai et al., 2014; Yamasaki et al., 2004). BPB, BPF and BPS are considered as alternatives to BPA and it is important to understand that whether these compounds are similar or more potent in endocrine disrupting activity than BPA.

In summary the current study provides information about the so called safer alternatives to BPA which have shown similar endocrine disturbances as BPA in animal studies. Most of these disturbances are either steroid or non-steroid pathways. In current study we reported that low concentration of these compounds for a long period of time can impair spermatogenic output and cause changes in the normal spermatogenesis in male rats. The hormonal levels were also altered which suggest that PBA analogues like BPB, BPF and BPS have endocrine disrupting properties by affecting the male reproductive functions in Sprague Dawley rats.

2. Material and methods

2.1. Animals

Male healthy rats (n = 91), weighing (30–40 g) were separated from their mothers on postnatal day 22 (PND 22) and were randomly divided into thirteen groups. Animals were kept in steel cages (7animals/cage) at temperature 22–25 °C and controlled light and dark cycle of 14–10 h light/dark. Animals were fed with laboratory feed (soy and alfalfa free) and water in poly sulfone bottles. All the experimental protocols were approved by the ethical committee of the department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

2.2. Experimental design

From PND 23, animals (n = 91) were allocated into thirteen different groups. First served as control and was provided with water containing (0.1% ethanol), while 2nd, 3rd and 4th groups were served with water containing 5, 25 and 50 μg/L BPA respectively. While 5th, 6th and 7th groups were served with water containing 5, 25 and 50 μg/L of BPB. Similarly, 8th, 9th and 10th groups were served with water containing 5, 25 and 50 μg/L of BPF and BPS was also given in water to 11th, 12th and 13th groups at a concentration of 5, 25 and 50 μg/L. All the bisphenols were dissolved in ethanol and the stock solution was diluted with water (final concentration of ethanol in the water was kept below 0.1%). Animals were provided with water alone or water with different concentrations of BPA, BPB, BPF and BPS for the period of 48 weeks. The duration of the exposure was selected according to the OECD test guideline 452 and the doses were selected on the basis of previous studies by (Ji et al., 2013) and (Chen et al., 2017). The BPA, BPB, BPF and BPS solutions in the water bottles was daily replaced with fresh solutions.

After the completion of the experimental period, animals were weighed, and seven animals per group were euthanized by cervical dislocation. Blood was collected from heart through cardiac puncture in heparinized syringes and was subjected to centrifugation at 3000 rpm for 15 min. Plasma was isolated and kept at −20 °C for hormonal assay. Reproductive organs (testis, epididymis, seminal vesicle and prostate) were dissected out and weighed for calculation of gonadosomatic index (GSI) and relative organs weight. Right epididymis and right testis were used for histology while left testis was used for DSP and biochemical analysis. Left epididymis was used for determination of sperm viability, motility and sperm count in the epididymis.

2.3. GSI and relative weight of organs

GSI is an important parameter used for estimation of gonadal maturity in the animals. GSI was obtained for each animal according to the formula used by Barber and Blake (2006).

$$\text{GSI} = \frac{\text{Gonadal weight (g)}}{\text{Body organs weight (g)}} \times 100$$

Relative weight of the organs was determined according to the following formula

$$\text{Relative organ weight} \frac{\text{Organ weight (mg)}}{\text{Body weight (g)}}$$

Relative weights of the organs were expressed as mg/g body weight.

2.4. Biochemical assays

2.4.1. Antioxidant enzymes

Tissues were collected and were processed for the antioxidant enzymes. Tissues were homogenized with automatic homogenizer in phosphate buffer saline and centrifuged at 30,000 g for 30 min. After the centrifugation the supernatant was removed and used for the hormonal analysis, protein estimation and antioxidant enzymes.

2.4.2. Catalase (CAT)

The catalase activity was determined by the method used by (Aebi, 1984) and the change in the absorbance due to H2O2 was measured in the testicular tissues. In this assay 50 ml homogenate was diluted in 2 ml of phosphate buffer with pH of 7.0. After mixing it thoroughly the absorbance was read at 240 nm with an interval of 15 s and 30 s. Change in the absorbance of 0.01 as unit/min was defined as one unit of CAT.

2.4.3. Super-oxidase (SOD)

Superoxide dismutase activity was estimated by the method developed by (Kakkar et al., 1984). In this assay the amount of chromogen formed was measured at 560 nm. The results were expressed in units/mg of protein.

2.4.4. Peroxidase (POD)

POD activity in homogenate was determined by spectrophotometric method of (Carlberg and Mannervik, 1975). In this assay 0.1 ml homogenate was mixed with 0.1 ml of guaiacol, 0.3 ml of H2O2 and 2.5 ml of phosphate buffer and the absorbance was read at 470 nm. Change in the absorbance of 0.01 as unit/min was defined as one unit of POD.

2.4.5. Lipid per oxidation by (TBARS)

Activity of T-BARS was determined in the homogenate by the method used by (Iqbal et al., 1996) and the results were expressed as TBARS/min/ml of plasma. In this assay 0.1 ml of homogenate was

mixed with 0.29 ml phosphate buffer, 0.1 ml of trichloroacectic acid, 1 ml of trichlorobarbituric acid followed by heating at 95 °C for 20 min and then shifted to ice bath before centrifuging at 2500 rpm for 10 min. The samples were read the help of spectrophotometer at 535 nm.

2.4.6. Reactive oxygen species (ROS)

The assay of reactive oxygen species (ROS) was done according to the method of (Hayashi et al., 2007) and for the presentation of mean values the assay was repeated multiple times. In this assay 5 ml of H2O2 standards and homogenate was mixed with 140 ml of sodium acetate buffer with pH 4.8 in 96 wells plate and incubated at 37 °C for 5 min. After the incubation 100 ml of DEPPD and ferrous sulphate mix sample was added in each well with a ratio of 1:25 and were incubated at 37 °C for 1 min. With an interval of 15 s for 3 min the absorbance was read at 505 nm at micro plate reader.

2.4.7. Total protein content

AMEDA Laboratory diagnostic kit was used for the determination of total protein in tissue. The results of protein were measured by plotting absorbance of the standard against samples. These values were expressed as mg/g of tissue.

2.5. Sperm motility and viability

Immediately after dissection, the cauda epididymis was cut slightly with a scissor in 0.5 ml pre-warmed (at 37 °C) phosphate buffered saline (pH 7.3) containing a drop of nigrosine stain. An aliquot of 50 µL was taken, placed on a pre-cleaned and warmed (at 37 °C) glass slide and was observed under a light microscope at 40X. A total of 100 sperm/sample were analyzed for motility by a technician blinded to the treatment groups. Each sample was analyzed three times and the average values were used as the total sperm motility. For viability, a drop of eosin and nigrosine was added to the sperm sample. A volume of 10 µL was placed on a pre warmed and cleaned glass slide and observed under a microscope at 100 X. Ten fields were analyzed by a person blinded to the treatment groups. A total of 100 sperm/field were checked for eosin staining and numbers of live and dead sperm were estimated. Each sample was repeated three times and average number was reported and expressed as percentage of live sperm.

2.6. Tissue histology

Testicular tissues (Testes and Epididymes) were fixed in formalin for 48 h. Dehydrated with different grades of Alcohol and cleared with help of xylene the paraffin sections (5 µm) were cut and stained with hematoxylin and eosin to assess standard histology and morphometry according to (Ullah et al., 2018). Testicular sections from 10 to 20 per group were digitized under Leica Microscope (New York Microscope company) equipped with digital camera (Canon, Japan).

For the morphometry the images were taken at 20x and 40x and the results were done with Image J software. Area of different sections was calculated with the method of (Jensen, 2013). From 20x images 30 picture per animal were selected and known area of different area of intestinal space, epididymis tubules and seminiferous tubules was measured by the software. Number of different cell types (spermatids, spermatogonia and spermatocytes) and area was calculated and comparison of different groups with control was done.

2.7. Sperm count and daily sperm production

Daily sperm production was done in the testicular tissues, with the help of rotostaor homogenizer (IKA-Werke, Staufen, Germany) the thawed samples were homogenized in 5 ml of solution which contained 0.5% NaCl and 5% triton X-100. The homogenized sample was diluted and samples were transpired to a neubar chamber and 19th stage spermatids were counted under microscope at 40X. Sperm count was done in the testicular tissues as the obtained values by the sperm count in the testes were divided by 6.3 (number of days the spermatids remain in seminiferous epithelium).

2.8. Hormonal analysis

Plasma testosterone and estrogen were determined by Enzymes linked immune sorbent assay (ELISA) kit purchased from Amgenix Inc. USA, while LH and FSH in plasma were determined by ELISA kits purchased from Reddot biotech.

2.9. Statistical analysis

Dunnet's multiple comparison test which followed (ANOVA) was used for the comparison of different groups with control using Graph Pad Prism software (version 5). Values were expressed as Mean ± SEM and were considered significant at $P < 0.05$.

3. Results

3.1. Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 µg/L) on initial and final body weight and body weight gain of male rats

Initial body weight, final body weight and body weight gain of the control animals and exposed group of different concentrations of BPA and its analogues BPB, BPF, BPS is presented in Table 1. At the start of the experiment all the animals were approximately of the same body weight, however, at the completion of the experiment the body weight of 50 µg/L BPA and its analogues BPB, BPF and BPS exposed groups were significantly high ($P < 0.05$) than control. On the other hand there was no significant difference observed in the final body weight of other treated groups with BPA and it analogues BPB, BPF and BPS when compared to the control. However, the body weight gain was also comparable to the control in the end of the 48 weeks experiment (Table 1).

3.2. Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 µg/L) on final body weight, GSI and absolute and relative weights of reproductive organs of male rats

Absolute and relative reproductive organs weight, GSI and body weight is represented in Table 2. Significant increase was observed in BPA, BPB, BPF and BPS 50 µg/L ($P < 0.05$) when compared to the

Table 1
Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 µg/L) on body weight gain of male rats.

Groups	Parameters		
	Initial Body weight (g)	Final Body Weight (g)	Body weight gain
Control	30.63 ± 0.38	541.11 ± 2.02	510.37 ± 2.25
BPA 5 µg/L	32.01 ± 0.31	537.81 ± 1.24	505.81 ± 0.96
BPA 25 µg/L	31.41 ± 0.50	538.40 ± 0.40	507.11 ± 0.44
BPA 50 µg/L	32.41 ± 0.40	549.40 ± 2.65*	517.11 ± 2.30
BPB 5 µg/L	31.98 ± 0.54	535.10 ± 1.44	503.018 ± 1.66
BPB 25 µg/L	31.41 ± 0.74	537.60 ± 1.02	506.21 ± 1.68
BPB 50 µg/L	32.61 ± 0.75	548.60 ± 1.83*	516.11 ± 2.09
BPF 5 µg/L	31.83 ± 0.95	537.80 ± 1.24	505.97 ± 1.12
BPF 25 µg/L	32.54 ± 0.86	538.40 ± 0.40	508.46 ± 1.20
BPF 50 µg/L	32.61 ± 0.67	548.20 ± 2.69*	515.61 ± 2.74
BPS 5 µg/L	32.61 ± 0.92	540.20 ± 2.35	506.41 ± 1.83
BPS 25 µg/L	33.03 ± 0.94	538.60 ± 0.50	507.77 ± 1.01
BPS 50 µg/L	33.26 ± 0.93	548.80 ± 2.28*	515.53 ± 2.98

Values are presented as Mean ± SEM.
*: Indicate significance at $p < 0.05$ vs control.

Table 2
Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 μg/L) on body and organs weight of male rats.

Groups	Parameters				
	Final body weight (g)	Paired testis (g)	GSI	Absolute Paired Epididymis (g)	Relative epididymis weight (mg/g)
Control	541.11	3.68 ± 0.08	0.69 ± 0.03	1.44 ± 0.03	2.65 ± 0.03
BPA 5 μg/L	537.82	3.54 ± 0.05	0.65 ± 0.02	1.42 ± 0.02	2.62 ± 0.02
BPA 25 μg/L	538.43	3.53 ± 0.05	0.66 ± 0.03	1.40 ± 0.03	2.61 ± 0.03
BPA 50 μg/L	549.41*	3.50 ± 0.03	0.64 ± 0.01*	1.39 ± 0.01	2.55 ± 0.02**
BPB 5 μg/L	535.12	3.53 ± 0.04	0.67 ± 0.04	142 ± 0.04	2.61 ± 0.03
BPB 25 μg/L	537.60	3.55 ± 0.05	0.66 ± 0.03	141 ± 0.03	2.60 ± 0.02
BPB 50 μg/L	548.60*	3.49 ± 0.03	0.65 ± 0.02*	140 ± 0.02	2.54 ± 0.01**
BPF 5 μg/L	537.80	3.54 ± 0.04	0.68 ± 0.04	142 ± 0.03	2.62 ± 0.04
BPF 25 μg/L	538.41	3.53 ± 0.05	0.66 ± 0.03	141 ± 0.04	2.61 ± 0.03
BPF 50 μg/L	548.22*	3.51 ± 0.03	0.64 ± 0.02*	142 ± 0.02	2.55 ± 0.02**
BPS 5 μg/L	540.20	3.55 ± 0.04	0.67 ± 0.04	143 ± 0.05	2.63 ± 0.03
BPS 25 μg/L	538.60	3.54 ± 0.05	0.68 ± 0.03	142 ± 0.04	2.60 ± 0.02
BPS 50 μg/L	548.81*	3.50 ± 0.03	0.65 ± 0.01*	141 ± 0.02	2.56 ± 0.02**

Values are presented as Mean ± SEM.
*: Indicate significance at $p < 0.05$ vs control.
**: Indicate significance at $p < 0.01$ vs control.

control. While, there was no significant difference in the other treatment groups observed when compared to the control. There was no significant difference observed in paired testis when comparison to the control after 48 weeks of exposure to different concentrations of BPA and its analogues BPB, BPF and BPS was done. GSI showed significant ($P < 0.05$) reduction in BPA, BPB, BPF and BPS 50 μg/L exposed groups. While there was no difference observed in the other treated groups when compared to control. There was also no significant difference observed in absolute paired testis of all the treated groups of bisphenols (BPA, BPB, BPF and BPS) when compared to the control, however, relative epididymis weight reduced significantly ($P < 0.01$) in BPA, BPB, BPF and BPS 50 μg/L treated groups. On the other hand, there was difference observed in the other treatment groups but that was not significant to the control (Table 2).

3.3. Effects of chronic exposure of different concentrations of BPS (5, 25 and 50 μg/L) on absolute seminal vesical weight, relative seminal vesical weight, absolute prostate weight and relative prostate weight of male rats

Seminal vesical weight and prostate weight after 48 weeks of exposure with different treatment groups and control is presented in Table 3. Significant reduction was observed in BPA 25 μg/L ($P < 0.05$), BPA 50 μg/L ($P < 0.01$) when compared to the control. Absolute seminal vesical was reduced significantly ($P < 0.05$, $P < 0.01$) in BPS 25 and 50 μg/L treated groups. Similarly, BPF treatment caused significant reduction ($P < 0.05$ and $P < 0.01$) at does levels of 25 and 50 μg/L. On the other hand, BPS 25 and 50 μg/L significantly reduced ($P < 0.05$ and $P < 0.01$) absolute seminal vesical weight; however other doses of BPA, BPB, BPF and BPS did not reduce absolute seminal vesical weight as compared to the control (Table 3).

Relative seminal vesical weight of different treatment groups of BPA and its analogues BPB, BPF and BPS is presented in Table 3. Significant reduction was observed in BPA 50 μg/L ($P < 0.01$) when compared to the control. Relative seminal vesical weight was reduced significantly ($P < 0.01$) in BPB 50 μg/L treated group. Similarly, BPF treatment caused significant reduction ($P < 0.01$) at 50 μg/L dose level. However, BPF 5 and 25 μg/L did not affect relative seminal weight significantly. BPS 50 μg/L relative seminal vesical weight was significantly reduced ($P < 0.01$), however, other doses did not reduce relative seminal vesical weight as compared to the control (Table 3).

Absolute and relative prostate weight after 48 weeks of exposure with different concentration of BPA and its analogues BPB, BPF and BPS is presented in Table 3. There was no significant difference observed in all the BPA and its analogues BPB, BPF and BPS treated groups as compared to the control. Prostate weight was observed to have reduced in some of the groups exposed to bisphenols but that reduction was not significant to the control (Table 3).

Table 3
Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 μg/L) on body and organs weight of male rats.

Groups	Parameters			
	Absolute seminal vesicle weight (g)	Relative seminal vesicle weight (mg/g)	Absolute prostate weight (g)	Relative prostate weight (mg/g)
Control	1.90 ± 0.04	3.55 ± 0.04	1.45 ± 0.03	2.71 ± 0.05
BPA 5 μg/L	1.88 ± 0.03	3.48 ± 0.03	1.42 ± 0.03	2.69 ± 0.04
BPA 25 μg/L	1.82 ± 0.02*	3.40 ± 0.04	1.46 ± 0.03	2.66 ± 0.03
BPA 50 μg/L	1.78 ± 0.03**	3.30 ± 0.03**	1.47 ± 0.04	2.65 ± 0.05
BPB 5 μg/L	1.86 ± 0.02	3.47 ± 0.03	1.43 ± 0.03	2.68 ± 0.03
BPB 25 μg/L	1.83 ± 0.03*	3.41 ± 0.04	1.45 ± 0.02	2.67 ± 0.04
BPB 50 μg/L	1.79 ± 0.04**	3.31 ± 0.02**	1.46 ± 0.04	2.65 ± 0.02
BPF 5 μg/L	1.86 ± 0.02	3.46 ± 0.04	1.42 ± 0.03	2.67 ± 0.04
BPF 25 μg/L	1.82 ± 0.02*	3.40 ± 0.03	1.44 ± 0.02	2.66 ± 0.03
BPF 50 μg/L	1.86 ± 0.03**	3.31 ± 0.03**	1.41 ± 0.04	2.64 ± 0.04
BPS 5 μg/L	1.87 ± 0.02	3.49 ± 0.02	1.44 ± 0.03	2.67 ± 0.03
BPS 25 μg/L	1.83 ± 0.03*	3.42 ± 0.04	1.46 ± 0.02	2.68 ± 0.04
BPS 50 μg/L	1.79 ± 0.03**	3.32 ± 0.03**	1.48 ± 0.04	2.64 ± 0.03

Values are presented as Mean ± SEM.
*: Indicate significance at $p < 0.05$ vs control.
**: Indicate significance at $p < 0.01$ vs control.
***: Indicate significance at $p < 0.001$ vs control.

3.4. Antioxidant enzymes, LPO and ROS after chronic exposure to different concentrations of BPA and its analogues BPB, BPF and BPS

Antioxidant enzymes reduced to a significant level while ROS and LPO levels increased in rats testicular tissues after chronic exposure to different concentrations of BPA and its analogues BPB, BPF and BPS as presented in Table 4. CAT activity was expressed as units/mg tissue and in BPA 25 μg/L and BPA 50 μg/L significant ($P < 0.05$) reduction was observed in exposed groups as compared to control. Similarly, significant reduction was also observed in BPB 25 μg/L ($P < 0.05$) and BPB 50 μg/L ($P < 0.01$) groups when compared to the control group. On the other hand, CAT activity was significantly reduced in BPF 50 μg/L ($P < 0.05$) as compared to control. In BPS exposed group only significant reduction was observed in BPS 50 μg/L ($P < 0.05$) when compared to the control group. While there was no significant difference observed in the other exposed groups of BPA, BPB, BPF and BPS as

Table 4
Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 μg/L) on oxidative stress in the testicular tissues of male rats.

Groups	Parameters				
	CAT (U/mg protien)	SOD (U/mg protien)	POD (U/mg protien)	LPO (U/mg protien)	ROS (U/mg protien)
Control	7.47 ± 0.15	32.34 ± 0.29	6.04 ± 0.15	7.72 ± 0.24	98.70 ± 0.29
BPA 5 μg/L	6.71 ± 0.41	32.09 ± 0.68	5.74 ± 0.07	7.62 ± 0.27	99.15 ± 0.18
BPA 25 μg/L	6.43 ± 0.25*	31.38 ± 0.43	5.60 ± 0.09*	7.73 ± 0.02	104.5 ± 1.67
BPA 50 μg/L	6.38 ± 0.25*	30.66 ± 0.33**	5.40 ± 0.10**	8.43 ± 0.07**	122.7 ± 3.53***
BPB 5 μg/L	7.11 ± 0.35	32.16 ± 0.30	5.65 ± 0.04	7.49 ± 0.07	98.35 ± 0.42
BPB 25 μg/L	6.38 ± 0.30*	31.34 ± 0.31	5.50 ± 0.13*	7.57 ± 0.08	105.0 ± 2.73
BPB 50 μg/L	6.09 ± 0.28**	30.81 ± 0.20*	5.42 ± 0.07**	8.60 ± 0.22**	122.6 ± 3.34***
BPF 5 μg/L	7.13 ± 0.13	32.32 ± 0.24	5.65 ± 0.05	7.38 ± 0.06	98.70 ± 0.42
BPF 25 μg/L	6.46 ± 0.27	31.14 ± 0.30	5.54 ± 0.11*	7.54 ± 0.09	105.4 ± 1.12
BPF 50 μg/L	6.17 ± 0.24**	30.42 ± 0.11**	5.41 ± 0.13**	8.59 ± 0.14**	122.0 ± 4.06***
BPS 5 μg/L	7.08 ± 0.26	32.59 ± 0.17	5.62 ± 0.09	7.48 ± 0.10	98.84 ± 0.40
BPS 25 μg/L	6.46 ± 0.20	31.63 ± 0.16	5.45 ± 0.09*	7.56 ± 0.08	105.4 ± 1.37
BPS 50 μg/L	6.36 ± 0.16*	30.57 ± 0.15**	5.44 ± 0.11**	8.60 ± 0.03**	121.5 ± 3.28***

Values are presented as Mean ± SEM.
*: Indicate significance at $p < 0.05$ vs control.
**: Indicate significance at $p < 0.01$ vs control.
***: Indicate significance at $p < 0.001$ vs control.

Table 5
Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 μg/L) on plasma testosterone and estradiol concentrations in male rats.

Parameters				
Groups	Testosterone (ng/ml)	Estradiol (pg/ml)	LH (ng/ml)	FSH (mIU/ml)
Control	12.02 ± 0.98	2.81 ± 0.33	1.79 ± 0.07	0.79 ± 0.07
BPA 5 μg/L	11.68 ± 0.43	3.64 ± 0.24	1.68 ± 0.08	0.75 ± 0.02
BPA 25 μg/L	10.61 ± 020	3.72 ± 0.40	1.55 ± 0.08	0.67 ± 0.04
BPA 50 μg/L	09.76 ± 0.36**	4.20 ± 0.34*	1.52 ± 0.03*	0.59 ± 0.05*
BPB 5 μg/L	11.05 ± 0.23	3.47 ± 0.19	1.62 ± 0.04	0.76 ± 0.07
BPB 25 μg/L	10.90 ± 0.21	3.93 ± 0.22	1.55 ± 0.03	0.63 ± 0.06
BPB 50 μg/L	09.36 ± 0.41***	4.55 ± 0.33**	1.48 ± 0.02*	0.58 ± 0.05*
BPF 5 μg/L	11.49 ± 0.37	3.53 ± 0.19	1.59 ± 0.08	0.73 ± 0.04
BPF 25 μg/L	10.43 ± 0.33	3.86 ± 0.26	1.54 ± 0.05	0.64 ± 0.01
BPF 50 μg/L	09.40 ± 0.05***	4.48 ± 0.29**	1.49 ± 0.07*	0.59 ± 0.02*
BPS 5 μg/L	11.39 ± 0.11	3.43 ± 0.31	1.63 ± 0.06	0.74 ± 0.03
BPS 25 μg/L	10.31 ± 0.63*	3.82 ± 0.16	1.56 ± 0.06	0.60 ± 0.02
BPS 50 μg/L	09.45 ± 0.33***	4.39 ± 0.29**	1.49 ± 0.02*	0.58 ± 0.03*

Values are presented as Mean ± SEM.
*: Indicate significance at $p < 0.05$ vs control.
**: Indicate significance at $p < 0.01$ vs control.
***: Indicate significance at $p < 0.001$ vs control.

compared to control.

SOD activity was expressed as (mU/mg protein) and in BPA 50 μg/L significant ($P < 0.01$) reduction was observed as compared to control. Similarly, BPB 50 μg/L exposed group caused significant ($P < 0.05$) reduction as compared to the control. On the other hand, BPF 50 μg/L significantly reduced ($P < 0.01$) SOD concentration in the rat testicular tissues. BPS high dose group 50 μg/L also ($P < 0.01$) reduced SOD concentration. However, 5 μg/L and 25 μg/L exposed groups did not show significant reduction in the SOD activity after chronic exposure with BPA, BPB, BPF and BPS.

POD activity was expressed as (U/mg protein) in the testis after chronic exposure, significant reduction in BPA 25 μg/L and 50 μg/L ($P < 0.05$ and $P < 0.01$) was observed as compared to the control. Significant reduction was observed in BPB 25 μg/L ($P < 0.05$) and BPB 50 μg/L ($P < 0.01$) when compared to the control. POD activity was reduced significantly ($P < 0.05$ and $P < 0.01$) in BPF 25 μg/L and BPF 50 μg/L treated groups. Similarly, BPS treatment caused significant reduction ($P < 0.05$ and $P < 0.01$) at dose levels of 25 and 50 μg/L. However BPA, BPB, BPF and BPS 5 μg/L did not affect POD activity significantly.

LPO activity in the different treatment groups and control after chronic exposure is presented in Table 4. Significant increase ($P < 0.01$) in BPA 50 μg/L was observed as compared to the control. All the high doses of BPB, BPF and BPS (50 μg/L) caused significant increase ($P < 0.01$) in the LPO activity as compared to control. However, there was no significant difference observed in 5 μg/L and 25 μg/L groups of BPA, BPB, BPF and BPS as compared to the control.

ROS in the testicular tissues of animals exposed to different concentrations of BPA, BPB, BPF and BPS for 48 weeks is presented in Table 4. Significant increase was observed in BPA 50 μg/L ($P < 0.001$) when compared to the control. ROS activity increased significantly ($P < 0.001$) in BPB 50 μg/L treated groups. Similarly, BPF treatment caused significant increase ($P < 0.001$) at 50 μg/L dose level. However, BPS 50 μg/L significantly increased ($P < 0.001$) ROS activity as compared to control. On the other hand, all the other doses (5 μg/L and 25 μg/L) of BPA, BPB, BPF and BPS did not cause significant reduction in the ROS activity as compared to the control.

3.5. Plasma testosterone, LH, FSH and estradiol concentrations in the animals after chronic exposure of 48 weeks to different concentrations of BPA and its analogues BPB, BPF and BPS

Plasma testosterone (ng/ml), Luteinizing hormone (ng/ml), Follicle-stimulating hormone (mIU/ml) and estradiol concentrations (ph/ml) are presented in Table 5. Significant reduction was observed in BPA 50 μg/L ($P < 0.01$) when compared to the control. Testosterone concentration reduced significantly ($P < 0.001$) in BPB 50 μg/L treated group. Similarly, BPF caused significant reduction ($P < 0.001$) at dose level 50 μg/L. On the other hand, BPS 25 μg/L and 50 μg/L significantly reduced ($P < 0.05$, $P < 0.001$ respectively) testosterone in the plasma, however other doses of BPA, BPB, BPF and BPS did not reduced plasma testosterone as compared to the control.

Plasma estradiol concentrations in the animals exposed to BPA 50 μg/L were significantly ($P < 0.05$) increased than control group. Estradiol concentration increased significantly ($P < 0.01$) in BPB 50 μg/L treated group. Similarly, BPF treatment caused significant increased ($P < 0.001$) at dose level of 50 μg/L, however, BPF 5 μg/L and 25 μg/L did not affect estradiol concentration significantly. On the other hand BPS 50 μg/L significantly increased ($P < 0.001$) estradiol concentration; however other groups did not increase estradiol concentration as compared to the control.

Plasma LH concentrations in the treatment groups were reduced as compared to the control (Table 5). Significant reduction was observed in BPA 50 μg/L ($P < 0.05$) when compared to the control. LH concentrations were reduced significantly ($P < 0.05$) in BPB 50 μg/L treated groups. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose level of 50 μg/L. BPS 50 μg/L significantly reduced ($P < 0.05$) plasma LH concentration, However other doses did not reduce plasma LH concentrations as compared to the control.

Plasma FSH concentrations in the treatment groups were found reduced as compared to the control group (Table 5). Significant reduction in plasma FSH levels ($P < 0.05$) was noted in the highest concentration (50 μg/L) exposed group of BPA when compared to the control. FSH concentration was reduced significantly ($P < 0.05$) in BPB 50 μg/L when compared to the control. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose level of 50 μg/L. On the other hand, PBS 50 μg/L significantly reduced ($P < 0.05$) FSH concentration in plasma. However, other treatment groups of BPA, BPB, BPF and BPS plasma FSH levels were reduced but were not statistically significant.

3.6. Sperm parameters, DSP and number of sperms in different parts of epididymis after chronic exposure to different concentrations of BPA, BPB, BPF and BPS

Exposure to different concentrations of BPA and its analogues BPB, BPF and BPS for 48 weeks caused no significant reduction in the percentage of motile sperm. However, Exposure to BPA highest concentration (50 μg/L) for 48 weeks caused significant ($P < 0.05$) reduction in motile sperm percentage but did not show effect on viable sperm percentage. Significant reduction was observed in BPB 50 μg/L ($P < 0.01$) when compared to control. Motile sperm percentage was reduced significantly ($P < 0.05$, $P < 0.01$) in BPF 25 and 50 μg/L. On the other hand, PBS 25 and 50 μg/L significantly reduced ($P < 0.05$, $P < 0.01$) percentage of motile sperms after exposure for 48 weeks of chronic exposure. However, in the different concentrations of BPA, BPB, BPF and BPS where no significant difference observed when compared to control (Table 6).

DSP in the different treatment groups and control is presented in Table 6. Significant reduction was observed in BPA 50 μg/L ($P < 0.01$) when compared to control. DSP was reduced significantly ($P < 0.01$) in BPB 50 μg/L treated group. Similarly, BPF treatment caused significant reduction ($P < 0.01$) at dose level of 50 μg/L. BPS 50 μg/L also caused significant reduction ($P < 0.01$) in the treated groups. On the other hand, BPA, BPB, BPF and BPS 5 and 25 μg/L treated groups did not affect DSP significantly.

Sperm number in caput epididymis was significantly reduced in the BPA 25 μg/L ($P < 0.05$) and BPA 50 μg/L ($P < 0.01$) exposed groups. Significant reduction was observed in BPB 25 μg/L ($P < 0.05$) and BPB 50 μg/L ($P < 0.01$) when compared to the control. Similarly, BPF treatment caused reduction ($P < 0.05$, $P < 0.01$) at dose levels of 25 and 50 μg/L. In BPS 25 and 50 μg/L caused significant reduction ($P < 0.05$, $P < 0.01$) in the caput epididymis sperm number when compared to the control. However, some of the BPA, BPB, BPF and BPS did not reduce sperm number in the caput epididymis as compared to the control.

Sperm number in the cauda epididymis in different treatment groups and control is presented in Table 6. Significant reduction was observed in BPA 50 μg/L treated group ($P < 0.05$) when compared to the control. Cauda epididymis sperm number was reduced significantly ($P < 0.05$) in BPB 50 μg/L treated group. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose level of 50 μg/L. BPS 50 μg/L also significantly reduced ($P < 0.05$) cauda epididymis sperm number as compared to control. On the other hand, there was no significant difference observed in BPA, BPB, BPF and BPS 5 and 25 μg/L treated groups when compared to the control.

3.7. Histological and planimetry changes of testicular tissue in adult male rats exposed to different concentrations of BPA, BPB, BPF and BPS for 48 weeks

Histological study of the microscopic slides of the testicular tissues revealed normal morphology of the structures in the control and 5 μg/L exposed groups. The seminiferous tubules were compactly arranged with sperm filled lumen and the interstitial space was relatively thin in these groups. In the groups exposed to 25 μg/L and 50 μg/L of BPA and its analogues BPB, BPF and BPS the tubules were relatively small with larger interstitial spaces and less filled lumen. Cellular arrest at spermatogoneal stage and at round spermatids were more evident in the highest concentration (50 μg/L) exposed group. In 25 μg/L exposed group, cellular arrest was observed but was less than 50 μg/L exposed group (Fig. 1).

Planimetry results showed significant ($P < 0.05$) reduction in the height of epithelium in the group exposed to 50 μg/L of BPA for weeks. Significant reduction was observed in BPB 50 μg/L ($P < 0.01$) when compared to the control. Epithelial height was reduced significantly ($P < 0.01$) in BPF 50 μg/L treated group. Similarly, BPS treatment caused significant reduction ($P < 0.05$) at dose level of 50 μg/L. However, BPA, BPB, BPF and BPS 5 and 25 μg/L groups did not affect epithelial height significantly. On the other hand, there was no significant difference observed in area of seminiferous tubules, area of interstitium and in diameter of seminiferous tubules of all treated groups of BPA, BPB, BPF and BPS as compared to the control (Table 7).

3.8. Number of different cells types in seminiferous tubules in the testis of adult rats exposed to different concentrations of BPA and its analogues BPB, BPF and BPS for 48 weeks

Number of different cells in the seminiferous tubules of male rats testis are presented in Table 8. Significant reduction in the number of spermatogonia was observed in the group exposed to BPA 50 μg/L ($P < 0.05$) than control. Significant reduction was also observed in BPB 50 μg/L ($P < 0.05$) treated group when compared to the control. Similarly, BPF treatment caused significant reduction ($P < 0.05$) at dose level of 50 μg/L. On the other hand, BPS 50 μg/L significantly reduced ($P < 0.05$) number of spermatogonia as compared to control. However, BPA, BPB, BPF and BPS 5 and 25 μg/L did not reduce significantly the number of spermatogonia as compared to control.

In the number of spermatocytes significant reduction was observed in BPA 50 μg/L ($P < 0.05$) when compared to the control.

Table 6
Effect of chronic exposure of different concentrations of BPA and its alternatives BPB, BPF and BPS (5, 25 and 50 μg/L) on sperm parameters and sperm number in epididymis of rats.

Groups	Parameters				
	Viable sperms (%)	Motile sperms (%)	DSP (x 106)	Caput epididymis sperm number (× 106/g organ)	Cauda epididymis sperm number (× 106/g organ)
Control	93.92 ± 0.48	79.56 ± 0.54	53.34 ± 0.6	303.16 ± 1.38	598.15 ± 2.46
BPA 5 μg/L	93.87 ± 0.65	77.72 ± 1.74	52.22 ± 0.3	296.62 ± 3.88	590.57 ± 0.22
BPA 25 μg/L	93.52 ± 0.92	77.01 ± 1.69	50.56 ± 1.4	291.78 ± 2.03*	589.28 ± 4.88
BPA 50 μg/L	92.01 ± 0.89	77.27 ± 0.89*	48.44 ± 0.3**	291.88 ± 4.11**	583.38 ± 1.64*
BPB 5 μg/L	93.95 ± 0.84	78.08 ± 0.68	52.34 ± 0.7	295.04 ± 2.10	592.18 ± 2.10
BPB 25 μg/L	93.13 ± 0.74	75.97 ± 0.51	51.04 ± 1.5	293.92 ± 2.04*	590.38 ± 5.06
BPB 50 μg/L	92.33 ± 0.86	74.17 ± 0.42**	48.32 ± 0.5**	290.16 ± 1.12**	580.98 ± 0.94*
BPF 5 μg/L	93.49 ± 0.97	78.33 ± 0.34	52.14 ± 0.6	295.14 ± 2.05	592.46 ± 2.02
BPF 25 μg/L	93.13 ± 1.09	75.33 ± 0.38*	50.68 ± 1.1	293.28 ± 0.75*	589.36 ± 2.66
BPF 50 μg/L	92.19 ± 0.91	74.70 ± 0.30**	48.58 ± 0.7**	288.86 ± 0.96**	583.14 ± 1.66*
BPS 5 μg/L	93.57 ± 1.07	78.12 ± 0.51	52.24 ± 0.5	295.52 ± 1.55	590.74 ± 5.07
BPS 25 μg/L	93.32 ± 1.01	75.27 ± 1.10*	50.32 ± 0.8	293.48 ± 1.77*	589.94 ± 4.88
BPS 50 μg/L	92.99 ± 0.97	74.28 ± 0.74**	48.22 ± 0.5**	291.12 ± 1.70**	584.64 ± 1.68*

Values are presented as Mean ± SEM.
*: Indicate significance at $p < 0.05$ vs control.
**: Indicate significance at $p < 0.01$ vs control.

Spermatocytes number was reduced significantly ($P < 0.05$) in BPB 50 μg/L treated group. Similarly, BPF 50 μg/L treatment caused significant reduction ($P < 0.05$) at dose level of 50 μg/L. BPS 50 μg/L treated group significantly reduced ($P < 0.05$) the number of spermatocytes when compared to the control. On the other hand, the other doses of BPA, BPB, BPF and BPS did not reduce number of spermatocytes as compared to the control.

Number of spermatids in different treatment groups and control is presented in Table 8. Significant reduction was observed in BPA 50 μg/L ($P < 0.01$) when compared to the control. Spermatids number reduced significantly ($P < 0.01$) in BPB 50 μg/L treated group. Similarly, BPF treatment caused significant reduction ($P < 0.01$) at dose level of

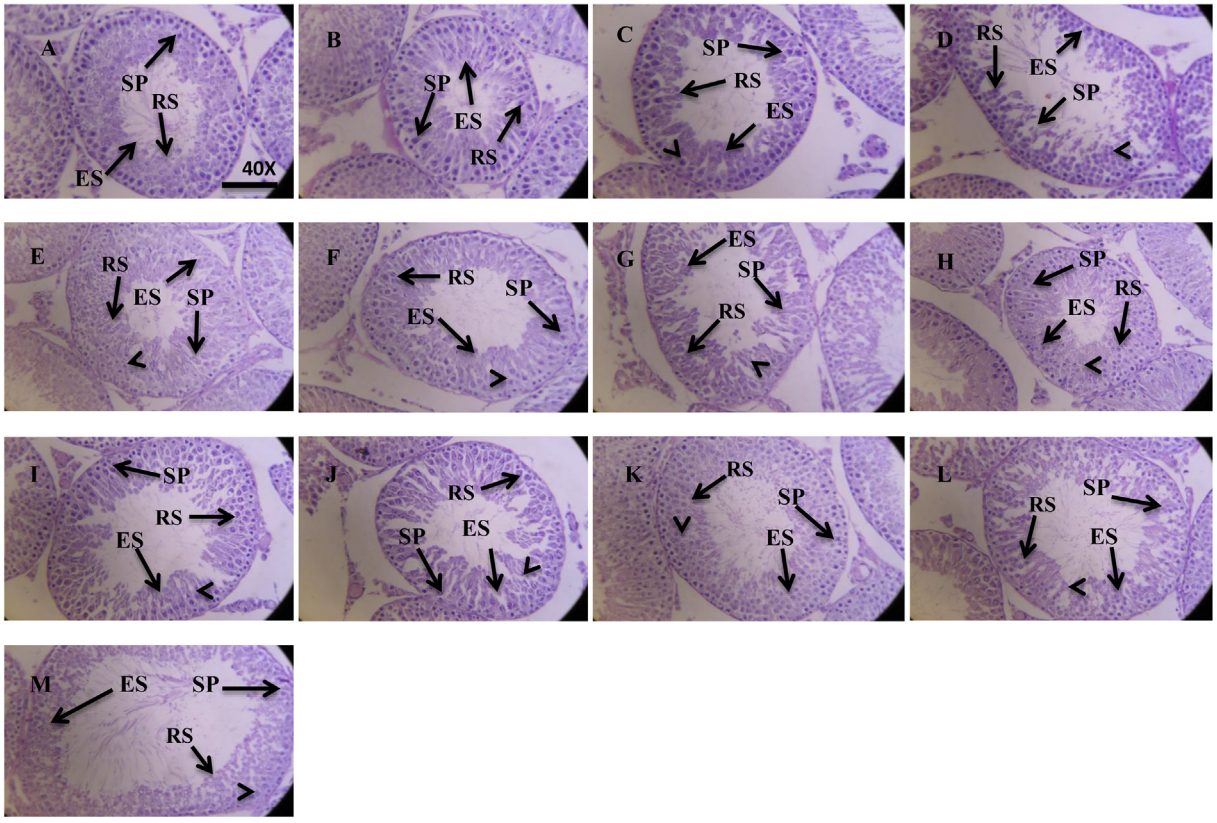

Fig. 1. Photomicrograph from testicular tissue showing (A) control; having thick epithelium with normal spermatogonia (SP), Round spermatids (RS), Elongated spermatids (ES) and filled lumen with sperm (B, C and D); BPA (5, 25 and 50 μg/L) treated presenting seminiferous tubules with epithelium (Line without arrow head) and spermatids (White arrow); (E, F and G) BPB (5,25 and 50 μg/L) treated presenting seminiferous tubules with epithelium (Line without arrow head) and elongating spermatids (White arrow); (H, I and J) BPF (5, 25 and 50 μg/L) treated presenting seminiferous tubules with epithelium (Line without arrow head) and elongating spermatids (White arrow); (K, L and M) BPS (5, 25 and 50 μg/L) treated presenting seminiferous tubules with epithelium (Line without arrow head) and spermatids (White arrow). H&E (40x).

Table 7
Effect of chronic exposure of different concentrations of BPA and its alternatives BPB, BPF and BPS (5, 25 and 50 μg/L) on planimetry of testis in rats.

Groups	Parameters			
	Area of seminiferous tubules (%)	Area of Interstitium (%)	Seminiferous tubule diameter (μm)	Epithelial height (μm)
Control	85.02 ± 1.95	16.42 ± 0.72	207.90 ± 1.77	71.22 ± 1.90
BPA 5 μg/L	82.64 ± 0.23	17.80 ± 0.95	201.08 ± 3.13	67.88 ± 1.02
BPA 25 μg/L	82.06 ± 0.67	16.22 ± 1.32	205.08 ± 1.55	65.74 ± 1.28
BPA 50 μg/L	82.17 ± 1.72	16.66 ± 1.38	203.97 ± 1.48	61.58 ± 2.17*
BPB 5 μg/L	82.73 ± 1.05	17.68 ± 0.38	205.87 ± 1.60	69.18 ± 1.29
BPB 25 μg/L	81.64 ± 0.56	15.90 ± 1.49	207.46 ± 1.47	68.13 ± 1.31
BPB 50 μg/L	83.71 ± 1.38	15.69 ± 1.37	203.24 ± 1.25	60.02 ± 2.72**
BPF 5 μg/L	84.58 ± 1.54	16.26 ± 1.63	204.81 ± 1.59	68.06 ± 2.10
BPF 25 μg/L	82.44 ± 0.71	15.65 ± 1.29	203.53 ± 1.72	66.35 ± 1.75
BPF 50 μg/L	84.46 ± 1.26	17.02 ± 1.51	205.46 ± 1.22	60.83 ± 2.15**
BPS 5 μg/L	83.51 ± 0.82	18.20 ± 0.52	205.24 ± 1.24	66.26 ± 2.65
BPS 25 μg/L	82.30 ± 0.69	17.86 ± 0.66	204.86 ± 1.58	64.44 ± 1.87
BPS 50 μg/L	83.28 ± 0.71	19.04 ± 0.78	204.35 ± 1.63	61.96 ± 2.72*

Values are presented as mean ± SEM.
*: Indicate significance at $p < 0.05$ vs control.
**: Indicate significance at $p < 0.01$ vs control.
***: Indicate significance at $p < 0.001$ vs control.

Table 8
Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 μg/L) on number of different cell types in the testis of rats.

Groups	Parameters		
	Spermatogonia (n)	Spermatocytes (n)	Spermatids (n)
Control	65.66 ± 0.62	77.10 ± 1.06	257.26 ± 1.79
BPA 5 μg/L	63.14 ± 0.75	75.40 ± 1.29	250.54 ± 2.67
BPA 25 μg/L	63.56 ± 0.83	73.32 ± 1.97	248.10 ± 2.71
BPA 50 μg/L	60.62 ± 0.72*	72.18 ± 1.20*	245.58 ± 2.42**
BPB 5 μg/L	63.98 ± 1.36	74.32 ± 0.94	250.32 ± 1.80
BPB 25 μg/L	63.68 ± 1.03	73.54 ± 1.41	248.36 ± 2.20
BPB 50 μg/L	61.26 ± 1.13*	71.82 ± 1.29*	245.40 ± 2.50**
BPF 5 μg/L	63.72 ± 1.13	73.64 ± 1.35	250.10 ± 2.87
BPF 25 μg/L	63.20 ± 1.16	72.64 ± 1.24	248.22 ± 2.34
BPF 50 μg/L	61.34 ± 0.84*	71.50 ± 1.26*	245.16 ± 1.97**
BPS 5 μg/L	63.40 ± 1.05	74.74 ± 1.30	250.04 ± 2.77
BPS 25 μg/L	63.64 ± 1.15	73.84 ± 1.23	248.32 ± 2.52
BPS 50 μg/L	61.58 ± 0.87*	72.12 ± 1.24*	244.02 ± 2.01**

Values are presented as mean ± SEM.
*: Indicate significance at $p < 0.05$ vs control.
**: Indicate significance at $p < 0.01$ vs control.

50 μg/L. BPS 50 μg/L group was also observed with significantly reduced ($P < 0.01$) number of spermatids as compared to the control. However, there was no significant difference observed in BPA, BPB, BPF and BPS 5, 25 μg/L groups when compared to the control.

3.9. Planimetry and morphological changes in the caput region of epididymis of rats exposed to different concentrations of BPA, BPB, BPF and BPS for 48 weeks

Epididymis Caput region Planimetry results did not show significant reduction in the tubular diameter in the groups exposed to different concentrations of BPA, BPB, BPF and BPS after 48 week chronic exposure. There was also no significant difference observed in the other parameters as lumen diameter, epithelial height and area covered with epithelium and lumen of different treatment groups when compared to the control (Table 9, Fig. 2).

There was very slight difference observed in the morphological difference of caput region of epididymis among the different treatment groups of BPA and its analogues BPB, BPF and BPS and control. In the different treatment groups of 50 μg/L of BPA, BPB, BPF and BPS slightly reduced number of sperm in the lumen was observed when compared to the control. There was no significant difference observed in the other exposed groups in comparison to the control (Fig. 2).

3.10. Planimetry and morphological changes in the cauda region of epididymis of rats exposed to different concentrations of BPA and its analogues BPB, BPF and BPS for 48 weeks

Planimetry of the cauda region of the epididymis showed no significant alteration in the tubular diameter in the groups exposed to different concentrations of BPA and its analogues BPB, BPF and BPS than control after 48 weeks of exposure. Similarly, other parameters like lumen diameter, epithelial height, area covered by epithelium and area covered by lumen did not show any significant alterations compared to the control (Table 10, Fig. 3). Morphological difference observed in the cauda region of epididymis showed only a slightly reduced number of sperms in the lumen of 50 μg/L exposed groups with different concentrations of BPA, BPB, BPF and BPS for 48 weeks of chronic exposure. No significant alterations were obvious in other groups in comparison with control (Fig. 3).

4. Discussion

A growing number of studies recently have reported the adverse toxic effects of bisphenol A involvement in many chronic diseases. Therefore, the concern of many environmental agencies and government security groups has led to the development of many substitutes for BPA such BPB, BPF and BPS. These all analogues leaching from plastic containers have been shown to a lesser extent; though it has been detected in a small amount in the food samples across the globe (Liao and Kannan, 2013, 2014a; b; Viñas et al., 2010; Yamazaki et al., 2015). Although there is very little data on the effects of low dose of BPA and its analogues BPB, BPF and BPS which are widely used to replace BPA. Widespread use of bisphenols caused growing concern over the adverse effects provoked by these substances on human health (Song et al., 2014). In vitro, in vivo studies and epidemiological surveys have shown that BPA and its analogues exhibits neurotoxic potential, hepatotoxic, cancer development risks and endocrine toxicity (Cabaton et al., 2009; Catanese and Vandenberg, 2016; Grignard et al., 2012; Rochester and Bolden, 2015; Soto et al., 2013; Ullah et al., 2018). There has been less attention given to BPA analogues and its toxicological effects on reproductive system.

The postnatal period is also a sensitive exposure period for certain endocrine disruptors to have a direct effect on the intra-testicular environment and adversely affect spermatogenesis. During the late fetal

Table 9
Effect of chronic exposure of different concentrations of BPA and its analogues BPB, BPF and BPS (5, 25 and 50 μg/L) on planimetry of caput epididymis in rats.

Groups	Parameters				
	Tubular diameter (μm)	Lumen daimeter (μm)	Epithelial height (μm)	Epithelium (%)	Lumen (%)
Control	366.40 ± 1.34	292.01 ± 2.76	34.05 ± 1.03	33.25 ± 2.37	70.75 ± 4.70
BPA 5 μg/L	358.80 ± 1.75	290.60 ± 2.61	33.40 ± 2.43	32.05 ± 1.50	69.75 ± 1.94
BPA 25 μg/L	356.20 ± 3.21	288.02 ± 1.90	30.04 ± 2.79	31.51 ± 0.49	68.55 ± 2.00
BPA 50 μg/L	357.20 ± 3.05	287.20 ± 2.22	29.40 ± 1.01	29.25 ± 2.49	64.25 ± 2.86
BPB 5 μg/L	359.04 ± 2.19	290.60 ± 1.70	33.04 ± 0.44	32.98 ± 1.06	69.55 ± 4.33
BPB 25 μg/L	358.40 ± 4.99	288.20 ± 1.48	31.40 ± 2.26	31.65 ± 0.48	68.75 ± 4.67
BPB 50 μg/L	357.80 ± 3.03	287.80 ± 0.95	30.75 ± 2.49	29.16 ± 1.13	65.75 ± 2.78
BPF 5 μg/L	358.40 ± 0.74	290.80 ± 1.96	33.05 ± 2.42	32.65 ± 2.17	67.95 ± 1.70
BPF 25 μg/L	359.20 ± 1.57	288.60 ± 0.24	31.40 ± 1.75	31.05 ± 1.83	65.25 ± 0.98
BPF 50 μg/L	356.80 ± 3.27	287.20 ± 2.47	30.05 ± 0.88	29.20 ± 1.13	64.35 ± 3.58
BPS 5 μg/L	357.60 ± 1.27	290.60 ± 2.98	33.60 ± 1.81	33.40 ± 1.58	68.95 ± 1.42
BPS 25 μg/L	355.80 ± 2.19	289.80 ± 4.63	31.20 ± 3.07	30.50 ± 2.39	66.05 ± 0.72
BPS 50 μg/L	354.40 ± 3.13	287.80 ± 3.02	30.40 ± 2.47	28.50 ± 1.49	65.75 ± 1.60

and early neonatal period, estrogenic substances can alter estrogen receptor (ER) expression in the testis, which will influence the ability of Leydig cells to function, and which will delay the eventual onset and progression of puberty (Sharpe et al., 2003). Bisphenols like BPA, BPB, BPF and BPS are toxicants that can cause a hypothyroid state in neonatal rats and is associated with increased number of Leydig cells, reduced size of Leydig cells, and decreased steroidogenic function of Leydig cells (Kim et al., 2001; Mendis-Handagama and Ariyaratne, 2004). Therefore, the reason of selecting rats of PND 20 was that to know about the effect of these bisphenols on the onset of puberty and how the later stage after puberty is disturbed after chronic exposure to low dose of these chemicals.

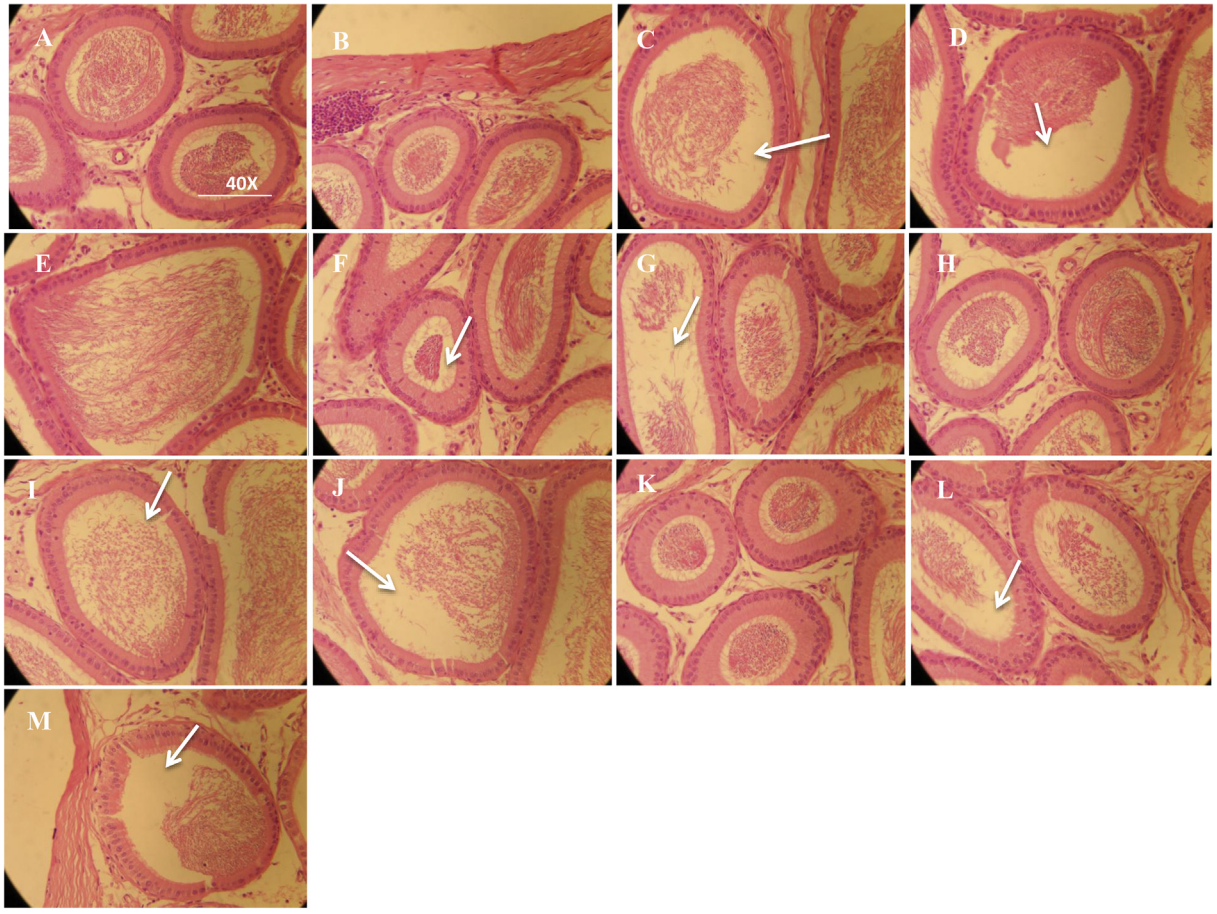

Fig. 2. Photomicrograph of caput epididymis tissue showing (A) control; with compact arrangement of caput tubules with sperm filled lumen (B) BPA (5 μg/L) exposed group, presenting normal caput tubules like in the control (C), BPA (25 μg/L) exposed group showing seminiferous tubules with less number of sperm in the lumen (Arrow) and (D) BPA (50 μg/L) exposed group presenting caput tubules with empty lumen (Arrow). Similarly, (E) BPB (5 μg/L) exposed group, presenting normal caput tubules, (F) BPB (25 μg/L) exposed group showing less number of sperms in the lumen, (G) BPB (50 μg/L) exposed group showing less number of sperms and empty lumen (Arrow). (H) BPF (5 μg/L) exposed group, presenting normal caput tubules, (I) (25 μg/L) exposed group showing seminiferous tubules with less number of sperm in the lumen (Arrow) and (J) BPF (50 μg/L) exposed group showing less number of sperms and empty lumen (Arrow). K, L BPS (5, 25 μg/L) exposed groups showing caput tubules with less number of sperms in the lumen and (M) BPS (50 μg/L) exposed group presenting less number of sperms and empty lumen. H& E (40x).

Table 10
Effect of chronic exposure of different concentrations of BPA and its analogues BPS, BPF and BPS (5, 25 and 50 μg/L) on planimetry of cauda epididymis in rats.

Groups	Parameters				
	Tubular diameter (μm)	Lumen diameter (μm)	Epithelial height (μm)	Epithelium (%)	Lumen (%)
Control	443.61 ± 1.67	415.60 ± 2.13	28.65 ± 1.05	33.25 ± 2.94	67.75 ± 1.97
BPA 5 μg/L	440.81 ± 0.72	412.60 ± 1.38	27.53 ± 1.46	31.51 ± 2.08	68.11 ± 0.88
BPA 25 μg/L	440.61 ± 3.91	411.11 ± 2.98	26.72 ± 0.86	28.91 ± 0.70	67.31 ± 1.68
BPA 50 μg/L	439.81 ± 2.32	410.10 ± 2.98	26.22 ± 1.75	27.75 ± 6.66	70.05 ± 1.69
BPB 5 μg/L	439.81 ± 0.95	413.40 ± 1.73	27.62 ± 1.45	29.51 ± 0.72	68.31 ± 2.27
BPB 25 μg/L	440.81 ± 2.95	415.60 ± 2.35	26.28 ± 1.68	27.25 ± 1.13	68.75 ± 1.87
BPB 50 μg/L	439.81 ± 3.11	414.60 ± 1.96	25.62 ± 2.10	26.75 ± 2.00	70.45 ± 1.27
BPF 5 μg/L	440.01 ± 0.54	414.40 ± 0.91	27.82 ± 2.45	31.51 ± 2.29	68.51 ± 2.00
BPF 25 μg/L	439.81 ± 1.22	413.20 ± 1.80	26.82 ± 2.39	29.75 ± 6.36	70.25 ± 1.67
BPF 50 μg/L	439.81 ± 1.13	413.12 ± 1.90	26.21 ± 1.00	27.51 ± 6.36	70.51 ± 3.55
BPS 5 μg/L	440.61 ± 2.13	414.13 ± 4.32	27.21 ± 2.19	29.51 ± 2.39	68.51 ± 2.54
BPS 25 μg/L	440.21 ± 1.05	413.80 ± 1.63	26.80 ± 3.10	27.75 ± 1.26	68.85 ± 1.17
BPS 50 μg/L	441.81 ± 1.75	413.40 ± 1.73	25.60 ± 3.24	27.51 ± 1.17	70.51 ± 3.55

In this study we have shown that BPB, BPF and BPS have many properties in common to BPA where we observed reduction in GSI, relative weights of reproductive organs, testosterone, LH and FSH concentrations and alterations in tissue histology in groups exposed to higher concentrations of BPA and its analogues BPB, BPF and BPS. Oxidative stress in the testicular tissue was induced and the DSP was reduced in the higher concentration exposed group than control. Our results were not very different from some of these studies done in past with BPA and its analogues where Meeker et al., 2009 in his study explained that BPA concentrations 1.3 (< 0.4–36.4) ng/mL in urine are in relation with reproductive hormones like testosterone and follicle stimulating hormone (FSH). Similarly, In another study Rubin 2011 explained the relation of BPA with reproductive hormones similar concentration with our results. On the other hand, Volkel et al., 2002 in

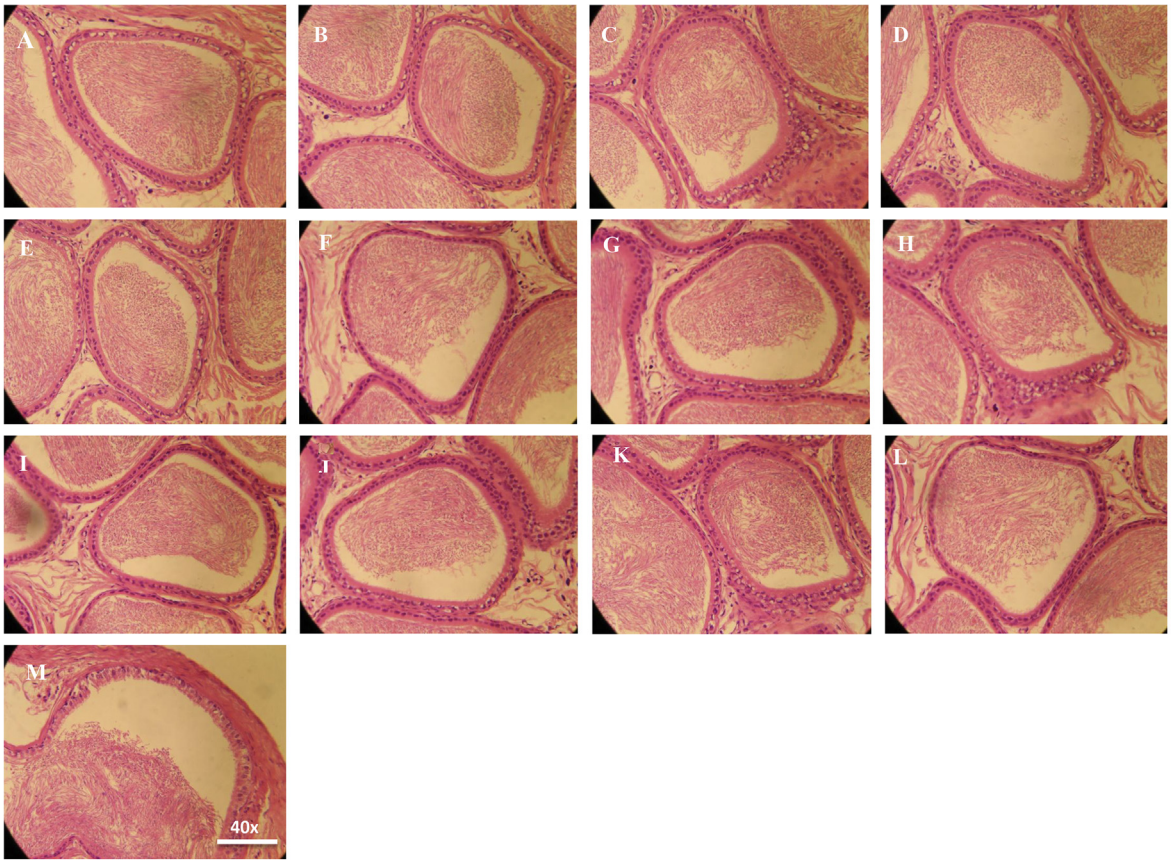

Fig. 3. Photomicrograph of cauda epididymis tissue showing (A) control; with compact arrangement of cauda tubules with sperm filled lumen (B) BPA (5 μg/L) exposed group, presenting normal caput tubules like in the control (C) BPA (25 μg/L) exposed group, presenting cauda tubules with sperm filled lumen (D) BPA (50 μg/L) exposed group presenting cauda tubules with less sperm in the lumen. Similarly, (E) BPB (5 μg/L) exposed group, presenting normal caput tubules like in the control (F) BPB (25 μg/L) exposed group, presenting cauda tubules with sperm filled lumen (G) BPB (50 μg/L) exposed group presenting cauda tubules with less sperm in the lumen. Likewise, (H)BPF (5 μg/L) exposed group, presenting normal caput tubules like in the control (I) BPF (25 μg/L) exposed group, presenting cauda tubules with sperm filled lumen (J) BPF (50 μg/L) exposed group presenting cauda tubules with less sperm in the lumen. In the same way, (K) BPS (5 μg/L) exposed group, presenting normal caput tubules like in the control (L) BPS (25 μg/L) exposed group, presenting cauda tubules with sperm filled lumen (M) BPS (50 μg/L) exposed group presenting cauda tubules with less sperm in the lumen. H&E (40x).

his study about BPA metabolic kinetics said that low dose (5 mg) of BPA in humans orally lead to altered reproductive hormones (Meeker et al., 2009; Rubin, 2011; Shi et al., 2015; Völkel et al., 2002).

In the present study hormones were disturbed of all the exposed groups to BPA and its analogues like BPB, BPF and BPS. Where we observed that both LH and FSH concentrations were inhibited and the concentration of testosterone had decreased in the exposed groups. However, the concentrations of estradiol in higher concentrations exposed groups had increased which suggests that either the gonadotropin secretions were inhibited at the level of pituitary or the secretions of GnRH from hypothalamus were affected which resulted in reduced levels of testosterone which needs further studies to be elucidated. This can also be because of disturbed testosterone machinery which produces testosterone and the disturbance resulted by prolonged oxidative stress in the testicular tissues. In the previous studies it was reported that oxidative stress induced by BPA and some of its analogues result into disturbed hormones in the different organisms (Feng et al., 2016; Hassan et al., 2012; Moghaddam et al., 2015; Naderi et al., 2014; Yang et al., 2017). In different studies previously it was reported that BPA and BPS exposure lead into oxidative stress in the peripheral blood mononuclear cells and testis and also lead into lipids and protein degradation in vitro (Michałowicz et al., 2015; Mokra et al., 2015; Ullah et al., 2016, 2017). The results of our study about inhibition of testosterone and anti-androgenic effects of these chemicals are in line with studies of Molina-Molina et al. (2013) an in-vitro study with low doses of BPA and BPS came across disturbed androgens levels after exposure to bisphenols and Rochester and Bolden 2015 also showed that bisphenol A analogues BPB and BPF have the potency to be in the same order of magnitude and in similar actions as BPA regarding androgens in both in vivo and in vitro studies (Molina-Molina et al., 2013; Rochester and Bolden, 2015). Testosterone reduced concentrations might be a result of suppression of GnRH transcripts in the hypothalamus which also suggest that suppressed GnRH lead in reduced gonadotropin secretion (Ji et al., 2013; Roelofs et al., 2015). However, increased estrogen levels seem to be due to estrogenic mode of action of bisphenol A and its analogues BPB, BPF and BPS (Liao and Kannan, 2013; Sui et al., 2012; Yamazaki et al., 2015).

Poor developments of reproductive organs lead into reduction in the daily sperm production, reduction in the GSI of male rats and alteration in the seminiferous tubules. The reduction of these parameters in our study were accompanied by arrest in spermatogoneal cells and round spermatids, which seem to have resulted because of reduced DSP, reduced number of sperm in the epididymis and epithelial height. Our results are in relation with multiple studies with BPA and some of its analogues where LH and FSH reduced levels supported the histological alterations in the testis and reduction in sperm production as in a by Brown et al. (2008) in the male rainbow trout exposed to 10 ng of EE2/l for 50 days showed altered reproductive hormones and it troubling embryonic aneuploidy whereas, Eladak et al., 2015 in his studies on BPA, BPF and BPS showed that 10 nmol/L-100 nmol/L of these compounds are involved in decreasing testosterone concentrations and alter physiological functions of reproductive organs (Brown et al., 2008; Chen et al., 2013; Eladak et al., 2015; Somm et al., 2009). Previous literature has also shown that estrogenic compounds do have effect on the reducing weight of the reproductive organs in the adulthood. The main reason for the reduction in weight and spermatogenesis is the presence of androgen and estrogen receptors in these organs that paly critical role in the spermatogenesis. On the other hand, gonadotropin receptor is also considered very important in the synthesis of androgens and spermatogenesis. It has been reported in several studies that any sort of alteration in these receptors lead into alteration in the testis physiology and spermatogenesis (Blake and Ashiru, 1997; Delfosse et al., 2014; Liang et al., 2016; Pelletier, 2000; Yang et al., 2017).

In the current study we observed that BPA and its analogues BPB, BPF and BPS at different concentrations not only resulted in potential hazardous effects on spermatogenesis but also lead into oxidative stress in the reproductive organs of male rats by reducing the DSP and altering seminiferous tubule epithelium. The results highlight the potential toxic effect of BPA and some of its analogues in different organisms tested in in-vitro and in-vivo studies where researchers observed the toxic effect of these compounds on male reproductive system (Chen et al., 2016b; Liang et al., 2016; Maćczak et al., 2016; Ullah et al., 2016, 2017, 2018; Zhang et al., 2016).

5. Conclusion

On the basis of the results from the present study, it can be concluded that exposure for a long period of time to low concentrations of BPA and its analogues BPB, BPF and BPS are capable of suppressing gonadotropins secretions from pituitary, exhibiting estrogenic and antiandrogenic effects in the mammals, inducing oxidative stress in the testicular tissues and affecting spermatogenesis by causing arrest at spermatogoneal stage as well as at the stage when spermatids can be seen. Further molecular studies need to be done to identify the exact mechanism of action of BPA and its analogues BPB, BPF and BPS through which it exhibits potential hazardous effects on the male reproductive tissues of mammals.

Declaration of interest

The authors report no declarations of interest.

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Transparency document

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Bisphenol A analogues bisphenol B, bisphenol F, and bisphenol S induce oxidative stress, disrupt daily sperm production, and damage DNA in rat spermatozoa: a comparative in vitro and in vivo study

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Abstract

Bisphenol A (BPA) is a well-known endocrine-disrupting chemical with estrogenic activity. The widespread exposure of individuals to BPA is suspected to affect a variety of physiological functions, including reproduction, development, and metabolism. Here we report the mechanisms by which BPA and three of its analogues bisphenol B (BPB), bisphenol F (BPF), and bisphenol S (BPS) cause generation of reactive oxygen species (ROS), sperm DNA damage, and oxidative stress in both in vivo and in vitro rat models. Sperm were incubated with different concentrations (1, 10, and 100 μg/L) of BPA and its analogues BPB, BPF, and BPS for 2 h. BPA and its analogues were observed to increase DNA fragmentation, formation of ROS, and affected levels of superoxide dismutase at higher concentration groups. In an in vivo experiment, rats were exposed to different concentrations (5, 25, and 50 mg/kg/day) of BPA, BPB, BPF, and BPS for 28 days. In the higher dose (50 mg/kg/day) treated groups of BPA and its analogues BPB, BPF, and BPS, DNA damage was observed while the motility of sperm was not affected.

Keywords

Bisphenol A, bisphenol B, bisphenol F, bisphenol S, oxidative stress, daily sperm production, DNA integrity

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Introduction

Bisphenol A (BPA; 2,2-bis (4-hydroxyphenyl) propane) is a high production volume chemical used for many plastic consumer products like food containers, water pipes, paper products, electronics, toys, and medical equipment (Vandenberg et al., 2009). Humans and animals are exposed to it via dietary and non-dietary pathways (Geens et al., 2012; Vandenberg et al., 2007). Presence of BPA in human urine, breast milk, umbilical cord, and placental tissues has been reported (Rochester, 2013). In both in vivo and in vitro studies, its effects on development and reproduction and on cardiovascular and neuronal networks have been documented (Bonefeld-Jørgensen et al., 2007; Richter et al., 2007). Exposure to BPA has lead regulations on its production, and in 2010, BPA use in baby bottles was banned in Canada and European Union (Crain et al., 2007; Chen et al., 2016; Vom Saal et al., 2007). Ban on BPA led to the production of alternative

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substances structurally similar to BPA (Rosenmai et al., 2014). Bisphenol F (BPF; 4,4′-methylenediphenol), bisphenol S (BPS; 4-hydroxyphenyl sulfone), and bisphenol B (BPB; 2,2-bis (4-hydroxyphenyl) butane) are among the main substitutes of BPA having broad range of applications (Cabaton et al., 2009; Chen et al., 2016; Matsushima et al., 2010; Naderi et al., 2014; Rosenmai et al., 2014).

Although studies on BPA analogues are limited in number, they are likely to cause cytotoxicity, reproductive toxicity, neurotoxicity, and endocrine disruption, as reported in several studies (Chen et al., 2016; Choi et al., 2004; Masuo and Ishido, 2011; Meeker et al., 2009; Ullah et al., 2018a). A study on BPA analogues showed that BPS and BPF have similar androgenic, anti-androgeic, estrogenic, and antiestrogen potencies (Rochester and Bolden, 2015). In some reports, it has been shown that BPA analogues may have endocrine-disrupting activities in different experimental models (Cano-Nicolau et al., 2016; Castro et al., 2015; Eladak et al., 2015; Feng et al., 2012; León-Olea et al., 2014; Le Fol et al., 2017; Negri-Cesi, 2015; Yang et al., 2014). Recently, few studies have shown that, in addition to BPA, the comparable concentrations of BPF, BPS, BPB in beverages and food products have been detected across the United States and in Asian countries (Liao and Kannan, 2013; Liao et al., 2012).

BPA and some of its analogues have a negative impact on the neuronal development and also interfere the normal functions of endocrine system (Cano-Nicolau et al., 2016; Liao et al., 2012; Molina-Molina et al., 2013). Recent studies showed that BPA analogues resulted in oxidative stress in the testes and altered reproductive functions in rats (Ullah et al., 2016, 2018a). Based on the previous study of oxidative stress-inducing potentials of BPA and its analogues BPB, BPF, and BPS (Ullah et al., 2018a), the present study aimed to investigate the effects of these compounds on DNA integrity in rat spermatozoa and oxidative stress in vitro and sperm DNA integrity and sperm production in vivo. The results of this study will help us understand the potential health implications of BPA and its alternatives and can reveal new information about the effects of these alternatives in animals.

Materials and methods

Animals and chemicals

Adult (70–80 days) male Sprague Dawley rats ($n = 117$) were obtained from the rodent colony of the Animal Sciences Department of Quaid-i-Azam University. Animals were kept in steel cages (seven animals per cage) under standard light conditions (light off from 19:00 to 05:00 h) at 22–25°C. The animals were fed with laboratory feed prepared as described elsewhere (Council, 1995) with slight modifications, that is, soy and alfalfa free, containing 20–25% protein, 4–7% fat, and 45–50% carbohydrates. Tap water was available ad libitum in polysulfone bottles free of BPA and analogues. Animal handling was approved by the Ethical Committee of the Animal Sciences Department (BAS 402006). BPA, BPB, BPF, and BPS (99% purity) were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Stock solutions of 2 g/L of BPA, BPB, BPF, and BPS (2 g/L) in ethanol were prepared and diluted with media/saline just before use.

In vitro experiment

According to the literature, male adult rats ($n = 26$) were used for obtaining sperm (Ullah et al., 2017; Xu et al., 2001). Animals were euthanized by cervical dislocation, and testicular tissues were removed and washed in saline. The dissected testes were cut into five equal parts and were processed in culturing tubes. Dulbecco's media containing penicillin, sodium bicarbonate, and streptomycin were mixed with 0, 1, 10, and 100 ng/mL of BPA and its analogues BPB, BPS, and BPF, and the culture tubes were incubated for 2 h in a CO_2 incubator. After 2 h of incubation, the tissues were removed from the culture media and washed with saline. Ninety milligrams of the cultured tissue was homogenized in 3 mL of phosphate buffered saline and centrifuged at 30,000 r/min for 30 min. The supernatant was discarded and the sperm pellets were used for various assays. The sperm pellets were suspended in 1-mL saline to be used for determination of antioxidant enzymes, and the remaining for comet assay and the values were expressed in 10^8 spermatocytes/mL.

Biochemical assays

Sperm pellets were thawed and centrifuged at 4°C for 10 min at $1000 \times g$. The supernatant was discarded and the samples were diluted in 50 mmol/L potassium phosphate buffer containing 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA) (pH 7.0) to a concentration of 1×10^8 spermatozoa/mL. Sperm were homogenized with ultrasonicator (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and the sonicated samples were used for the assay of reactive

oxygen species (ROS), lipid peroxidation (LPO), and superoxide dismutase (SOD).

Reactive oxygen species

The assay for determination of ROS was done according to the method of Hayashi et al. (2007) with slight modifications. For the presentation of mean values, the assay was repeated multiple times. Standards of H_2O_2 (30% w/w, Sigma Aldrich, St. Louis, Missouri, United States) were prepared with serial dilutions (0.23, 0.46, 0.92, 1.87, and 7.50 mg H_2O_2), and a volume of 5-µl standards or homogenate were diluted with 140 µL of 0.1 mol/L sodium acetate buffer with pH (4.8) in 96 wells plate and incubated at 37°C for 5 min. A volume of 100 µL of mixed solution of *N*,*N*-diethyl-para-phenylenediamine and ferrous sulfate (1:25) were added in each well and incubated at 37°C for 1 min. Absorbance was obtained at 505 nm using a microplate reader for 180 s with 15 s interval. Standard curve was plotted and concentrations of ROS in unit/10^8 spermatocytozoa/mL were reported. One unit of ROS was considered equivalent to levels of hydrogen peroxide in the sample (1 unit = 1.0 mg H_2O_2/L).

Thiobarbituric acid reactive substances

The amount of thiobarbituric acid reactive substances (TBARS) as an index of LPO was assessed by measuring the peroxidation reaction between Thiobarbituric acid (TBA) and malonaldehyde (MDA) at high temperature and low PH. The reaction results in the production of pink color that can be measured by obtaining the absorbance at 535 nm using spectrophotometer against a reagent blank (Iqbal et al., 1996; Ohkawa et al., 1979). Sperm were centrifuged at 1000 x g for 10 min, the supernatant was discarded, and cells were diluted to the 10^8 sperm/1 ml and were homogenized through ultrasonication. The homogenized sample was mixed with 0.01 mL Tris-HCl buffer (150 mM, pH 7.1), and 0.01 mL ferrous sulphate (1.0 mM), 0.01 mL ascorbic acid (1.5 mM), and 0.06 mL H_2O were mixed and incubated at 37°C for 15 min; 10% w/v trichloroacetic acid was added to stop the reaction. TBA (0.2 mL; 0.375% w/v) was added and the sample was incubated at 100°C for 15 min. Finally, samples were centrifuged at 1000 × *g* for 10 min. The amount of MDA formed in each sample was estimated by measuring optical density at 532 nm. Results were expressed as nmol of TBARS/min/10^8 spermatozoa at 37°C using a molar extinction coefficient of 156 mM/cm and was expressed in nmol of TBARS/10^8 spermatozoa.

Superoxide dismutase

SOD activity was estimated by the method developed by Kakkar et al. (1984). In this assay, the amount of chromogen formed was measured by recording the absorbance at 560 nm using a spectrometer. The results were expressed in mU/10^8 of spermatozoa.

After obtaining the results from the in vitro study, an in vivo study was carried out to check the possible hazardous potentials of BPA and its analogues BPB, BPF, and BPS.

In vivo experiment

A total of 91 adult male Sprague Dawley rats were divided equally into 13 groups, with 7 animals per group, and were randomly assigned to different treatments as follows. The control rats received daily gavage of 2-mL water containing 0.1% ethanol as vehicle. The other groups received ethanolic solutions of BPA, BPB, BPF, and BPS at final concentrations of 5, 25, and 50 mg/kg/day in 2 mL water for 28 consecutive days. Subchronic exposure for 28 days was based on the enhanced OECD test guideline (Yamasaki et al., 2002a, 2002b). The selection of BPA, BPB, BPF, and BPS doses was according to the previous studies (Ullah et al., 2018a, 2018b). On the 29th day, all the animals were killed by decapitation; testis and epididymis were dissected out and processed for determination of sperm motility, daily sperm production (DSP), and DNA damage.

Sperm motility

Immediately after dissection, the cauda epididymis was cut slightly with a scissor in 0.5-mL prewarmed (at 37°C) phosphate buffered saline (pH 7.3) containing a drop of nigrosine stain. An aliquot of 50 mL was taken, placed on a pre-cleaned and warmed (at 37°C) glass slide, and was observed under a light microscope at 40×. A total of 100 spermatocytes/sample were analyzed for motility by a technician blinded to the treatment groups. Each sample was analyzed three times, and the average value was used as the total sperm motility.

Daily sperm production

Prior to the homogenization, frozen testicular tissues were thawed at room temperature, tunica albuginea

was removed, and the parenchyma was weighed and homogenized in 5 mL of solution, containing 0.9% NaCl and 0.5% Triton X-100 for 30 s using a rotor-stator homogenizer (IKA-Werke, Staufen, Germany). The homogenate was diluted fivefold, a volume of 20 mL homogenate was transferred to a Neubauer chamber, and 19th stage spermatids were counted under a light microscope at 40× magnification. A total of three readings were taken for calculation of the average number of spermatids in each sample. These values were used to obtain the number of spermatids per testis and were divided by 6.3 (number of days the spermatids remain in seminiferous epithelium) to determine DSP.

$$\mathrm{DSP} = \frac{Y}{6.3}$$

Assessment of DNA damage

DNA damage of individual spermatozoa was assessed using a modified neutral comet assay according to (Boe-Hansen et al., 2005). Sperm from the cauda epididymis were collected in phosphate buffered saline (pH 7.3) and diluted to the concentration of 10^5 spermatozoa/mL. Similarly, sperm from the in vitro experiments were centrifuged at 1000 xg for 10 min. The supernatant was discarded and the sperm pellet was diluted with phosphate buffered saline to a concentration of 10^5 spermatozoa/mL. Shortly, a layer of regular melting point agarose was applied to the slides and cover slipped. Slides were placed at low temperature until the gel solidified. The coverslips were removed and a second layer of 85-μL low melting point agarose (65 μL of 1% low melting point agarose and 20 μL of sperm suspension from in vivo and in vitro experiments) was spread on top of the first layer. Slides were cover slipped and allowed to solidify. Lysis of cells was carried out by placing the slides in freshly prepared cold lysis buffer (pH 10.3, 2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris Base, 1% (w/v) Triton X-100) for 24 h. After washing with distilled water (20 min each) three times, the slides were placed in an electrophoresis tray containing neutral electrophoresis buffer (54 g/L Tris base, 27.5 g/L boric acid, 0.5 mol/L EDTA, pH 7.4). Electrophoresis was performed for 20 min at 25 V (0.71 V/cm). The slides were air dried, covered with aluminum foil, and kept at 5°C overnight. The slides were rehydrated with distilled water for 60 min. The water was then drained from the slides and 1.0 mL SYBR Red (1: 10,000 dilution) was applied to the slides for 60 min. The slides were rinsed with distilled water and cover slips ($22 \times 50\ \mathrm{mm}^2$) were placed. The DNA comets were visualized using 20× objective lens attached to a Nikon Optiphot-2 epifluorescence microscope (Plan Fluor, Nikon, UK). Pictures were taken through an intensified solid-state CCD camera (Sony CCD-IRIS, Minato, Tokyo, Japan) attached to the microscope and connected to a Pentium 1133 MHZ PC, which provided images on the Comet assay II software (Perceptive Instruments, UK). The software provides a rectangular measurement frame, and the stained DNA were scored for Comet tail length (TL), tail moment (TM) as distance between centers of mass % tail DNA, tail intensity (TI), and head intensity (HI). The measurements were calculated for two duplicate sample slides, with 50 DNA scored per slide.

Statistical analysis

The statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, California, USA). A one-way analysis of variance (one-way ANOVA) was used to analyze the differences between treatments within each experiment control. Dunnett's multiple comparison tests were used to compare sample groups with control. A value of $p < 0.05$ was considered statistically significant. For all values, means ± standard errors of means (SEM) were calculated.

Results

In vitro sperm incubation with different concentrations of BPA and its analogues BPB, BPF, and BPS on SOD and LPO

Antioxidant activities of SOD and LPO were determined after incubation of sperm with different concentrations of BPA and its analogues BPB, BPF, and BPS. SOD activity showed significant increase ($p < 0.05$) in BPA 100 μg/L (5.65 ± 0.29 mU/10^8 Spermatozoa) as compared to the control (3.71 ± 0.10 mU/10^8 Spermatozoa/mL). Significant increase was observed in BPB 100 μg/L ($p < 0.01$) when compared to the control. Similarly, BPF 100 μg/L also caused significant increase ($p < 0.01$) when compared to control. In BPS, 100 μg/L significant increase ($p < 0.05$) was observed as compared to control. On the other hand, total SOD activity in other treated groups was also increased (around 2–4 mU/10^8

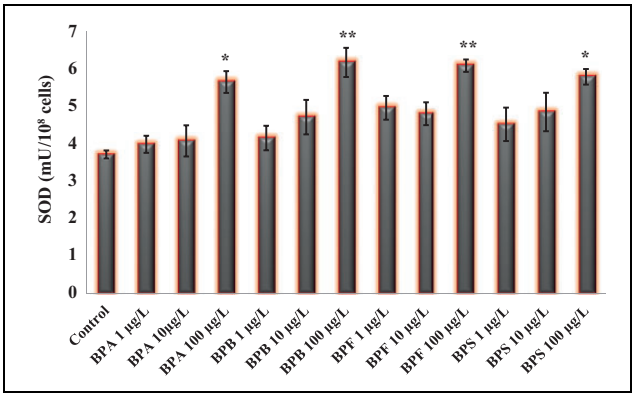

Figure 1. In vitro effect of BPA, BPB, BPF, and BPS on SOD activity in rat sperm after 2 h of incubation. SOD activity measured in control and BPA, BPB, BPF, and BPS (1, 10, and 100 µg/L) treated rat sperm groups. Results are expressed as mean ± SEM ($n = 7$ for each condition) and presented as SOD (mU/10^8 cells). *,**,***Significant results ($p < 0.05$, $p < 0.01$) are indicated: versus control. SOD: superoxide dismutase; BPA: bisphenol A; BPB: bisphenol B; BPF: bisphenol F; BPS: bisphenol S; SEM: standard errors of mean.

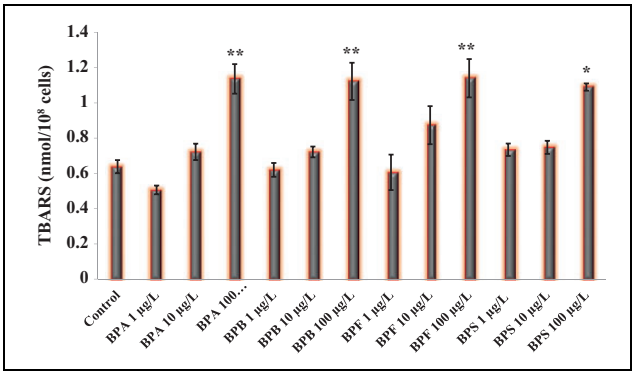

Figure 2. Effect of BPA, BPB, BPF, and BPS on TBARS in rat sperm after 2 h of incubation in vitro. TBARS measured in control and BPA, BPB, BPF, and BPS (1, 10, and 100 µg/L) treated rat sperm groups are expressed as mean ± SEM ($n = 7$ for each condition) and presented as TBARS (nmol MDA/10^8 cells). *,**,***Significant results ($p < 0.01$, $p < 0.01$) are indicated: versus control. MDA: malonaldehyde; TBARS: thiobarbituric acid reactive substances; BPA: bisphenol A; BPB: bisphenol B; BPF: bisphenol F; BPS: bisphenol S; SEM: standard errors of mean.

Spermatozoa/mL) but was not significantly different from control (Figure 1).

TBARS activity in different treatment groups and control is presented in Figure 2. Significant increase was observed in BPA 100 µg/L ($p < 0.01$) when compared to control. The measured TBARS activity in 100 µg/L BPA-treated group was 1.13 ± 0.04 nmol MDA/10^8 spermatozoa, while in control group it was

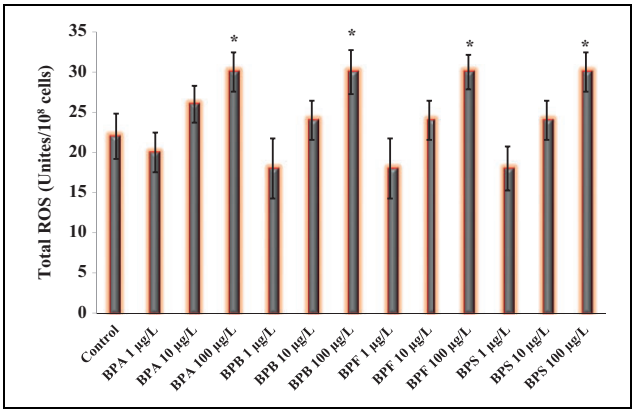

Figure 3. Effect of BPA and its analogues BPB, BPF, and BPS on ROS in rat sperm after 2 h of incubation in vitro. ROS measured in control and BPA, BPB, BPF, and BPS (1, 10, and 100 µg/L) treated rat sperm groups are expressed as mean ± SEM ($n = 7$ for each condition) and presented as ROS (units/10^8 cells). *,**,***Significant results ($p < 0.05$) are indicated: versus control. BPA: bisphenol A; BPB: bisphenol B; BPF: bisphenol F; BPS: bisphenol S; SEM: standard errors of mean; ROS: reactive oxygen species.

as 0.63 ± 0.03 nmol MDA/10^8 spermatozoa. Similarly, in BPB and BPF, 100 µg/L groups significant increase ($p < 0.05$) was observed in as compared to control. TBARS activity increased significantly ($p < 0.05$) in BPS 100 µg/L-treated group. Though, the TBARS activity of other treatment groups of BPA and its analogues BPB, BPF, and BPS had increased but that increase was not significant as compared to control.

Reactive oxygen species

Oxidative stress was checked in the samples by measuring ROS in the treated groups with BPA and its analogues BPB, BPF, and BPS presented in Figure 3. In control group, values of ROS were around 22.0 ± 2.82 unit/10^8 spermatozoa/mL, which were lower to the spermatozoa exposed BPA at 100 µg/L 30.0 ± 2.45. While the values of BPB and BPF 100 µg/L also increased significantly ($p < 0.05$) compared to control, and the values in these two groups were 30.0 ± 2.74 and 30.0 ± 2.16. Similarly, ROS levels also increased ($p < 0.05$) in treated group with BPS at 100 µg/L as 30.0 ± 2.45 compared to control. There was no significant change observed in ROS levels of BPA and its analogues in different groups 1–10 µg/L in comparison with control.

DNA damage

DNA damage in the spermatozoa was measured by comet assay and is presented in Table 1. The

Table 1. Average values of rat sperm DNA damage in control and sperm incubated with different concentrations of BPA, BPB, BPF, and BPS (1, 10, and 100 μg/L) for 2 h in vitro.[a]

Groups	Parameters		
	Number of comets/ 100 cells	Tail moment (μm)	Tail DNA (%)
Control	14.62 ± 0.16	6.33 ± 0.15	14.54 ± 0.25
BPA 1 μg/L	15.08 ± 0.31	6.97 ± 0.30	14.02 ± 0.28
BPA 10 μg/L	13.97 ± 0.16	7.78 ± 0.18	15.96 ± 0.34
BPA 100 μg/L	16.41 ± 0.30[b]	8.44 ± 0.70[b]	17.34 ± 0.21[b]
BPB 1 μg/L	13.57 ± 0.60	6.92 ± 0.16	15.12 ± 0.28
BPB 10 μg/L	14.79 ± 0.20	7.52 ± 0.22	15.82 ± 0.18
BPB 100 μg/L	16.06 ± 0.72[b]	8.16 ± 0.36[b]	17.12 ± 0.16[b]
BPF 1 μg/L	13.81 ± 0.12	6.16 ± 0.16	13.98 ± 0.22
BPF 10 μg/L	14.35 ± 0.14	8.24 ± 0.13	14.92 ± 0.22
BPF 100 μg/L	16.58 ± 0.82[b]	8.06 ± 0.24[b]	17.51 ± 0.18[b]
BPS 1 μg/L	13.48 ± 0.16	6.41 ± 0.17	13.82 ± 0.30
BPS 10 μg/L	14.83 ± 0.26	7.06 ± 0.55	14.62 ± 0.34
BPS 100 μg/L	16.65 ± 0.60[b]	8.48 ± 0.22[b]	16.98 ± 0.06[b]

BPA: bisphenol A; BPB: bisphenol B; BPF: bisphenol F; BPS: bisphenol S; SEM: standard errors of mean.
[a]Values are expressed as mean ± SEM.
[b]Significance at $p < 0.05$ versus control.
[c]Significance at $p < 0.01$ versus control.
[d]Significance at $p < 0.001$ versus control.

underlying principle of comet assay is the ability of damaged DNA fragments to migrate during electrophoresis. The results show non-significant difference in DNA fragmentation in spermatozoa nuclei of BPA and its analogues BPB, BPF, and BPS (1–10 μg/L) treated groups as compared to the control after 2 h of in vitro exposure but there was significant ($p < 0.05$) increase of DNA fragmentation in spermatozoa in BPA and its analogues BPB, BPF, and BPS groups at 100 μg/L observed as compared to control group.

In vivo effects of subchronic exposure of BPA, BPB, BPF, and BPS in rats

Sperm motility in all the groups treated with different doses of BPA, BPB, BPF, BPS, and control showed no significant difference in the total sperm motility of treated groups as compared to control. The motile sperm number in BPA groups was 85.8 ± 1.70%, 86.7 ± 1.23%, and 84.5 ± 1.22% in 5, 25, 50 mg/kg/day as compared to control which was 87.8 ± 1.09. While in the treatment groups with BPB 5, 25 and 50 mg/kg/day, the number of motile sperm was 86.9 ± 1.16, 85.8 ± 1.11, and 84.0 ± 1.69. Similarly, BPF 5, 25, and 50 mg/kg group showed similar number of motile sperm as 85.4 ± 1.43, 84.18 ± 1.43, 83.8 ± 1.76. BPS 5, 25 and 50 mg/kg/day group also showed similar number of motile sperm as 85.1 ± 0.87, 84.9 ± 0.28, 83.7 ± 0.92 in treatment groups.

Significant difference ($p < 0.05$) was observed in BPA 50 mg/kg/day group when compared to control. While, there was no significant difference observed in BPA 5 and 25 mg/kg/day treated groups when compared with control. On the other hand, there was also no significant difference observed in BPB 5 and 25 mg/kg/day group as compared to control. Although in BPB 50 mg/kg/day, there was significant difference ($p < 0.05$) observed as compared to control. BPF and BPS 50 mg/kg/day treated groups showed significant difference of ($p < 0.05$) in comparison to control. There was no significant reduction observed in groups treated with BPF and BPS (5 and 25 mg/kg/day) as compared to the control group.

DNA damage is presented in Table 2 of different concentrations of BPA and its analogues BPB, BPF, and BPS after 28 days of exposure. In the tail DNA, there was significant difference ($p < 0.01$) observed in BPA 50 mg/kg/day group as compared to control. In BPA 5 and 25 mg/kg/d groups, there was no significant difference observed. There was also no significant difference observed in BPB 5 and 25 mg/kg/day groups as compared to control while an increase ($p < 0.05$) was observed in BPB 50 mg/kg/day group. On the other hand, there was also no significant difference observed in BPF 5 and 25 mg/kg/day groups while significant increase ($p < 0.01$) was observed in BPF 50 mg/kg/day as compared to control. BPS 50 mg/kg/day group showed significant ($p < 0.05$) increase as compared to control, while there was no significant difference observed in BPS 5 and 25 mg/kg/day treated groups when compared to the control.

Discussion

BPB, BPF, and BPS have been used as alternative to BPA (Rosenmai et al., 2014). Data from many agencies that monitor the environment have shown that these chemicals are going to become a serious threat to both human and animal life and are going to become the most concerned environmental pollution and food contaminant in the future (Liao and Kannan, 2013). In the present study, BPA, BPB, BPF, and BPS comparative toxic effects on the antioxidant enzymes of sperm (Mu et al., 2018; Qiu et al., 2018), DSP, DNA damage, and ROS were evaluated. In the in

Table 2. Average values of sperm DNA damage in control and BPA and its analogues BPB, BPF, and BPS (5, 25, and 50 mg/kg/day) treated rats after 28 days of treatment.[a]

Groups	Parameters		
	Number of comets/ 100 cells	Tail moment (μm)	Tail DNA (%)
Control	18.28 ± 0.38	11.28 ± 0.75	18.44 ± 0.63
BPA 5 mg/kg	17.71 ± 0.91	11.93 ± 0.20	20.54 ± 1.01
BPA 25 mg/kg	20.85 ± 1.77	12.16 ± 0.30	19.31 ± 1.47
BPA 50 mg/kg	23.78 ± 0.74[b]	14.62 ± 1.14[b]	25.71 ± 1.11[b]
BPB 5 mg/kg	18.81 ± 0.66	11.84 ± 0.29	19.03 ± 0.68
BPB 25 mg/kg	22.63 ± 0.92	13.28 ± 0.27	22.41 ± 1.19
BPB 50 mg/kg	23.16 ± 0.55[b]	14.77 ± 1.02[b]	25.93 ± 0.67[b]
BPF 5 mg/kg	19.95 ± 0.68	12.52 ± 0.38	17.85 ± 1.13
BPF 25 mg/kg	22.47 ± 0.84	13.74 ± 0.27	21.27 ± 0.80
BPF 50 mg/kg	23.21 ± 1.32[b]	14.64 ± 0.24[b]	24.57 ± 0.53[b]
BPS 5 mg/kg	19.15 ± 0.70	12.92 ± 0.36	18.21 ± 1.40
BPS 25 mg/kg	22.42 ± 0.67	12.88 ± 0.35	22.98 ± 0.28
BPS 50 mg/kg	23.58 ± 0.89[b]	14.82 ± 0.33[b]	25.32 ± 0.82[b]

BPA: bisphenol A; BPB: bisphenol B; BPF: bisphenol F; BPS: bisphenol S; SEM: standard errors of mean.
[a]Values are expressed as mean ± SEM.
[b]Significance at $p < 0.05$ versus control.
[c]Significance at $p < 0.01$ versus control.
[d]Significance at $p < 0.001$ versus control.

vitro study, the production of ROS and increased activity of LPO indicated oxidative stress in rat sperm after exposure to BPA and its analogues. BPA, BPB, BPF, and BPS exposure in vivo lead to substantial damage to DNA and reduced levels of DSP.

BPA and other phenolic compounds have been shown to neutralize ROS by many studies (Kourouma et al., 2015; Lee et al., 2013; Huc et al., 2012; Hulak et al., 2013). Oxidative stress also affects the function of sperm by damaging lipids in the sperm plasma membrane (Ullah et al., 2017, 2018a; Zalata et al., 2004). The current study results showed that SOD activity was high in sperm samples incubated with BPA, BPS, BPF, and BPS groups, which seem to be due to activation of body defense mechanism of antioxidant enzyme to reutilize free radicals generated by ROS. In previous studies, it was also reported that BPA and some of its analogues incubated for 2 h also increased the levels of SOD in testicular tissues (Ullah et al., 2016, 2017, 2018a). Some other studies on BPA and BPS exposure also increased the activity of SOD by inducing oxidative stress in the sperm and reproductive tissues (Hulak et al., 2013; Potts et al., 2000; Ullah et al., 2017) leading to produce high levels of LPO in the testicular tissues. In the present study, levels of ROS and TBARS were also observed high in the groups treated with BPA, BPB, BPF, and BPS. Stress in the sperm cells in groups treated with BPA, BPB, BPF, and BPS resulted in an increased oxidative stress and high activity of ROS which is supported by previous in vitro and in vivo studies (Lee et al., 2013; Liang et al., 2016; Ullah et al., 2017, 2018a, 2018b). In the previous studies, BPS and BPA exposure also increased the activity of SOD and induced oxidative stress in the sperm cells, reproductive tissues, and blood cells (Dong et al., 2018; Maćczak et al., 2017; Manfo et al., 2014; Rhee and Rhee, 2016).

In the in vivo study, a significant reduction in DSP and increase in DNA damage was observed in the sperm cells of all treated groups with BPA and its analogues BPB, BPF, and BPS. Previously, several studies have shown that BPA and some of its analogues exposure led into reduced testosterone levels, increased levels of estrogen, reduce number of eggs and pups, and modified transcripts of GnRH (Ahsan et al., 2018; Feng et al., 2012; Ji et al., 2013; Roelofs et al., 2015; Ullah et al., 2017, 2018a, 2018b). BPA has already shown that it mimics estrogen and have anti-androgenic effects that result in the reduction of DSP and suppress the levels of testosterone hormone (Ahsan et al., 2018; Grignard et al., 2012; Sakaue et al., 2001; Ullah et al., 2016, 2017, 2018a). On the basis of the above studies, it is possible that the anti-androgenic effects of BPA, BPB, BPF, and BPS might have led to the reduction in DSP in the present study.

BPA and some of its analogues have also been reported to be genotoxic and, in the in vitro and in vivo studies, they have also induced apoptosis (Barbonetti et al., 2016; Irvine et al., 2000; Mokra et al., 2015; Rahman et al., 2015; Yin et al., 2016). The present in vivo and in vitro studies suggest that BPA and its analogues BPB, BPF, and BPS exposure reduce the number of sperm and also damage the sperm DNA that suggest the relation of sperm motility and DNA damage.

Conclusion

The in vivo and in vitro studies on BPA and its analogues BPB, BPF, and BPS exhibit altered DSP, affected sperm quality and DNA damage. The results of the present study provide evidence of the genotoxic potential as well as oxidative stress-inducing ability of BPA and its analogues BPB, BPF and BPS in rat sperm, both in vivo and in vitro conditions, which might be due to the generation of ROS and LPO in sperm. Our findings not only provide new insights into toxicity but also show that these alternatives are not that safe and further studies shall be carried out to reveal the mechanisms underlying toxicity in the reproductive tissues.

Declaration of Conflicting Interests

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Effect of bisphenol F, an analog of bisphenol A, on the reproductive functions of male rats

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Abstract

Objective: Bisphenol A (BPA) is a monomer primarily used in the production of polycarbonate plastic and epoxy resins. Bisphenol F (BPF) is apparently the main BPA replacement that is used increasingly. BPF has been detected in canned food, thermal paper receipts, and soft drinks. In the present experiment, we did both in vitro and in vivo studies to evaluate the effect of low and high-dose BPF exposures on testosterone concentration, oxidative stress, and antioxidants activity in reproductive tissues of male rats.

Methods: Adult (80–90 days old) male Sprague Dawley rats ($n = 36$) obtained from the rodent colony of Animal Sciences Department of Quaid-i-Azam University. The direct effects of BPF on the antioxidant enzymes and testosterone secretion were measured in vitro and in vivo studies. In an in vivo experiment, adult male Sprague Dawley rats ($n = 42$) were exposed to different concentrations of bisphenol F (1, 5, 25, and 50 mg/kg/d) for 28 days. Various biochemical parameters were analyzed including the level of catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), reactive oxygen species (ROS), and lipid peroxidation (LPO). Moreover, sperm motility, daily sperm production (DSP), comet assay, and histological analysis were performed.

Results: In vitro study showed that BPF exposure significantly ($p < 0.05$) induced oxidative stress biomarkers, i.e., ROS and LPO, while it did not change antioxidant enzyme and testicular testosterone concentration. Whereas, an in vivo study revealed that BPF induced dose-dependent effect and high-dose (100 mg/kg) exposure of BPF significantly reduced tissue protein ($p < 0.05$) content, CAT ($p < 0.001$), SOD ($p < 0.05$), and POD ($p < 0.05$) levels while significantly ($p < 0.05$) augmented ROS and lipid peroxidation. Furthermore, BPF reduces testosterone, LH, and FSH secretion in a dose-dependent manner. Significant ($p < 0.001$) reduction in plasma and intra-testicular testosterone, LH, and FSH was noticed at 100 mg/kg BFP dose. High-dose exposure reduces spermatogenesis.

Conclusion: BPF showed an antagonistic effect on male reproductive hormones and induce alterations in testicular morphology. Increased oxidative stress and decreased testicular antioxidant status might be the underlying mechanism of BFP-induced testicular toxicity.

Keywords: Bisphenol F, Male reproductive system, Reproductive toxicity, Antioxidant enzymes, Oxidative stress

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Introduction

Bisphenol A (BPA) is a monomer primarily used in the production of polycarbonate plastics and epoxy resins [1, 2]. BPA used in thermal papers does not bind covalently with macromolecules of polymer, and with ease migrates into food and beverages [2]. BPA's possible route of exposure in humans is food, drinking water, and beverages except for occupational exposure [3]. Studies have shown that beverages in cans are more contaminated with BPA and its analog than those packed in glass containers [2]. Following the restrictions on the use of BPA in the canning industry moving towards safer alternatives of BPA [4], among the BPA alternatives, a large class of compounds shares chemical and physical properties with BPA with variable toxicity and higher estrogenic activities. Among this group of compounds, bisphenol F (BPF) is apparently the main replacement to BPA. BPF has been detected in canned food, thermal paper receipts, and soft drinks. BPF has also exhibited endocrine-modulating capabilities and its toxicity has also shown genotoxic effects, carcinogenic potencies, reproductive complacencies, and oxidative stress [5–8].

BPF has a wide spectrum use in the plastic industry and it has been detected in 55 of the 100 tested urine samples with a concentration of 0.08 μg/L [9]. Similar, concentrations of BPF were observed from 600 urine samples collected in the US from 2000 to 2014 with a concentration of 0.15–0.54 μg/L [10, 11]. HepG2 cell line treated with BPF resulted in oxidative stress and endocrine activities. In another study on HepG2 cells, it was observed that BPF has a higher affinity for ER α and β receptors than that of other bisphenols [12–14]. Significantly less information about potential adverse health outcomes is available about BPF regarding its toxicity. Similar to BPA, BPF is an endocrine-disrupting chemical and displays hormonal activity, with similar average estrogenic, androgenic, and antiestrogen potencies across different in vitro assays. BPF differentially affects signaling pathways involved in lipid metabolism and adipogenesis and causes DNA damage. The present study aimed to examine the possible effects of BPF exposure on the reproductive system of mammals by using rats as an animal model.

Methods

Chemicals

Bisphenol F (BPF) with 99% purity was purchased from Santa Cruz Biotechnologies, USA. For the in vitro experiment, different materials as fetal bovine serum, penicillin/streptomycin, and Dulbecco's modified Eagle's medium (DMEM) were obtained from Thermos Fisher Scientific (Waltham, MA, USA). CAT, N-acetyl-L-cysteine (NAC) and H_2O_2, Ca^{2+}, Mg^{2+}, Hank's balanced salt solution (HBSS) were bought from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Sprague Dawley adult male rats (age 80–90 days) were obtained from the animal facility of Quaid-i-Azam University, Islamabad. Prior to the start of the experiment standard laboratory conditions were maintained. Animals were fed with laboratory feed and tap water was available freely for the animals. Protocols of handling of the animals were approved by the animal sciences department ethical committee.

Experimental design

For bisphenol F (BPF) exposure on male rats, different experiments were conducted. Firstly, we conducted an in vitro experiment in which the direct effects of BPF on the levels of antioxidant enzymes and different concentrations of testosterone in the testis of rats were tested. While on the results of the in vitro study, an in vivo study was conducted in which the effects of different concentrations of BPF on the reproductive system of male rats were evaluated through subchronic study.

In vitro studies

In the in vitro study, a total of ($n = 36$ and $n = 6$ animals per group) Sprague Dawley male adult rats were used. In order to investigate the direct effects of BPF on the antioxidant enzymes and testosterone production, an in vitro study was conducted. In this study, different doses of BPF (0, 1, 10, 25, 50, and 100 ng/ml) were prepared in ethanol which was in accordance with [15, 16]. The culturing of testicular tissues was done by the method of [16] with little modifications. Healthy male rats were euthanized and the testes were removed and placed in clean Petri dishes and were cut in equal parts and placed in culture tubes. Culture media containing Dulbecco's, penicillin, sodium bicarbonate, and streptomycin were mixed with 0, 1, 10, 25, 50, and 100 ng/ml of BPF with the method explained elsewhere by [17]. All the culture tubes containing media, testicular tissues, and BPF different concentrations were incubated in a carbon dioxide (CO_2) incubator for 2 hours. After the incubation period, all the incubated tissues were washed with saline and homogenized in 30 ml of phosphate buffer saline (PBS) and centrifuged at 30,000 for 30 min. Then the supernatant was collected and stored at − 80 °C for further investigation.

In vivo study

Adult male Sprague Dawley rats ($n = 42$) were divided into six groups ($n = 7$/group) by randomization procedures explained elsewhere [18]. All the animals were exposed to different concentrations (1, 5, 25, 50, and 100 mg/kg body weight/ day) of BPF for 28 days.

Group 1: Control received saline

Group 2: Administration of BPF at a dose of 1 mg/kg body weight/day

Group 3: Administration of BPF at a dose of 5 mg/kg body weight/day

Group 4: Administration of BPF at a dose of 25 mg/kg body weight/day

Group 5: Administration of BPF at a dose of 50 mg/kg body weight/day

Group 6: Administration of BPF at a dose of 100 mg/kg body weight/day

No mortality was recorded during the period of experimentation. At the end of the experiment (on the 29th day), animals were euthanized and different organs were dissected and stored at − 80 °C for different tests. Blood was collected and centrifuged at 3000 rpm for 10 mins and plasma was separated and stored at − 20 °C for hormonal and different biochemical analysis by the researcher blind to the treatment groups. The reproductive organs as testicular tissues (left testis and left epididymis) were weighed and processed for antioxidant enzymes while right testis (transverse sections) and right epididymis were fixed in 10% formalin for histological analysis as explained by [19].

Biochemical analysis

Tissues collected from both in vitro and in vivo studies were further processed for the antioxidant enzymes and oxidative stress markers. Tissues were homogenized with an automatic homogenizer in phosphate buffer saline (PBS) and centrifuged at 30,000 rpm for 30 mins. After the centrifugation, the supernatant was removed and used for the hormonal analysis, protein estimation, and antioxidant enzymes [17, 19].

Catalase (CAT)

Afsar et al.'s method was used to determine the catalase (CAT) activity [20], and the change in the absorbance was measured in the tissues. In this assay 50 ml, the homogenate was diluted in 2 ml of phosphate buffer with a pH of 7.0. After mixing it thoroughly the absorbance was read at 240 nm with an interval of 15 s and 30 s. Change in the absorbance of 0.01 as unit/min was defined as one unit of CAT.

Superoxide dismutase (SOD)

Afsar and colleagues method was used to determine the superoxide dismutase (SOD) activity [21]. In this assay, the amount of chromogen formed was measured at 560 nm. The results were expressed in units per milligram of protein.

Peroxidase (POD)

Peroxidase (POD) activity in the homogenate was determined by the spectrophotometric method of Carlberg and Mannervik, [22]. In this assay, the homogenate was mixed with 0.1 ml of guaiacol, 0.3 ml of H_2O_2, and 2.5 ml of phosphate buffer and the absorbance was read at 470 nm. Change in the absorbance of 0.01 as unit per minute was defined as one unit of POD.

Lipid peroxidation (LPO)

The activity of lipid peroxidation by T-BARS was determined in the homogenate by the method used by Iqbal and coworkers [23] and the results were expressed as TBARS per minute per milliliters of plasma. In this assay, 0.1 ml of homogenate was mixed with 0.29 ml phosphate buffer, 0.1 ml of trichloroacetic acid, and 1 ml of trichlorobarbituric acid followed by heating at 95 °C for 20 min and then shifted to an ice bath before centrifuging at 2500 rpm for 10 min. The samples were read with the help of spectrophotometer at 535 nm.

Reactive oxygen species (ROS)

The assay of reactive oxygen species (ROS) was done according to the method of Hayashi et al. [24]. In this assay, 5 ml of H_2O_2 standards and the homogenate was mixed with 140 ml of sodium acetate buffer with pH 4.8 in 96-well plates and incubated at 37 °C for 5 min. After the incubation, 100 ml of DEPPD and ferrous sulphate mix samples were added in each well with a ratio of 1:25 and were incubated at 37 °C for 1 min. With an interval of 15 s for 3 min, the absorbance was read at 505 nm at microplate reader.

Protein estimation

Determination of total protein content in tissues was done following a commercial diagnostic kit (AMEDA Labordiagnostik Laboratory, Austria) protocol. The results of protein were measured by plotting absorbance of the standard against samples. These values were expressed as milligram per gram of tissue.

Hormonal analysis

Quantitative EIA kits were used for the measurement of testosterone (BioCheck Inc., USA Catalog No. BC-1115), luteinizing hormone (LH) (BioCheck Inc., USA Catalog No.BC-1031), and follicle-stimulating hormone (FSH) (BioCheck Inc., USA Catalog No.BC-1029) concentrations in the tissues and the assays were performed by the instructions with the kits. All the above assays were repeated with both inter- and intra-assay variations for more and precise results.

Tissue histopathology

Testicular tissues (testis and epididymis) were fixed in formalin for 48 h, dehydrated with different grades of alcohol, and cleared with the help of xylene. The paraffin sections (5 μm) were cut and stained with hematoxylin and eosin for histology and morphometry. Transverse sections (10–20/group) of testicular tissues were examined under a Leica

Microscope (New York Microscope Company) equipped with a digital camera (Canon, Japan).

For the morphometry, the images were taken at × 20 and × 40, and the results were done with Image J software. Area of different sections was calculated with the method of Jensen et al. [25]. From × 20 images, 30 pictures per animal were selected and the known area of different areas of intestinal space, epididymis tubules, and seminiferous tubules was measured by the software. The number of different cell types (spermatids, spermatogonia, and spermatocytes) and the area were calculated, and comparison of different groups with control was done.

Statistical analysis

All parameters of data points showed normal distribution and hence were reported as mean ± SEM and difference was considered significant at $P < 0.05$. One way ANOVA followed by Dunnet's multiple comparison tests was used for the comparison of different groups with control using Graph Pad Prism software.

Results

Bisphenol F in vitro effects on the testicular tissues antioxidants, ROS and testosterone secretions in the rat testis

Antioxidant enzymes, i.e., CAT, POD and SOD, oxidative stress markers, i.e., reactive oxygen species (ROS) and TBARS, were determined in the testicular tissues after 2 hours incubation with different concentrations of BPF (Table 1). There was no significant difference observed in the CAT, POD, and SOD activity in any of the BPF-treated groups as compared to the control.

Reactive oxygen species and LPO are considered important oxidative stress markers. In BPF 50 ng/ml and 100 ng/ml treated groups, significant ($P < 0.05$) increases in LPO were observed as compared to the control. However, there was no significant increase observed in the low-dose-treated groups as compared to the control. Similarly, there is a dose-dependent augmentation in ROS levels in different treatment groups. In BPF 25 ng/ml and 50 ng/ml, significant ($P < 0.05$) increase in ROS was noticed, whereas in BPF 100 ng/ml, marked ($P < 0.01$) increase in ROS was examined as compared to the control group. Low doses of BPF did not induce any change in ROS level compared to the control group.

The levels of testosterone in the testis after 2 hour incubation with the treatment of different concentrations of BPF decreased but that difference was not significant as compared to control (Table 1).

Bisphenol F different concentration effects on the body weight gain and testicular weight after sub-chronic administration

BPF exposure in male rats for 28 days did not show any significant change in the body weight of all treated groups as compared to the control. There was also no significant difference observed in the left testis and right testis of all the treated groups with BPF when compared to the control (Table 2).

Bisphenol F different concentration sub-chronic effects on the biochemical parameters of rat testis

Antioxidant enzymes in the testicular tissues after 28 days of different concentrations of subchronic exposure to BPF and control are presented in Table 2. There was no significant difference observed in the activity of SOD when different treatment groups of BPF were compared with control. On the other hand, there was a significant difference observed in the activity of POD when different treated groups of BPF were compared with control. A significant reduction was observed in BPF 5 mg/kg ($P < 0.05$), BPF 25 mg/kg ($P < 0.01$), BPF 50 mg/kg ($P < 0.05$), and BPF 100 mg/kg ($P < 0.05$) when compared to the control.

BPF treatment caused significant ($P < 0.05$) reduction in CAT activity at doses of 5, 25, and 50 mg/kg treated groups as compared to control. Similarly, BPF treatment caused significant ($P < 0.01$) decline in CAT activity at dose levels of 100 mg/kg treated groups.

ROS and LPO level in different treatment groups are presented in Table 2. LPO which is a well-known oxidative

Table 1 In vitro effect of Bisphenol F (BPF) on antioxidant enzymes and testosterone secretion in rat testis

Groups (n = 6/group)	Parameters					
	CAT (u/mgProtein)	POD (nmole)	SOD (u/mgprotein)	LPO (nM TBARS/min/mg Tissue)	Total ROS (U/g tissue)	Testosterone (ng/g tissue)
Control	9.53 ± 0.43	8.12 ± 0.60	10.51 ± 1.78	31.11 ± 1.81	29.00 ± 2.32	52.32 ± 2.02
BPF 1 ng/ml	8.39 ± 0.53	7.43 ± 0.79	11.99 ± 2.01	24.17 ± 1.11	35.60 ± 2.35	49.04 ± 2.45
BPF 5 ng/ml	7.94 ± 0.49	6.92 ± 1.13	12.08 ± 2.23	40.07 ± 2.56	29.60 ± 2.08	46.48 ± 1.60
BPF 25 ng/ml	7.95 ± 0.85	6.46 ± 1.28	13.66 ± 2.10	39.21 ± 2.85	38.60 ± 2.47*	44.71 ± 2.31
BPF 50 ng/ml	7.80 ± 1.29	7.06 ± 1.85	13.15 ± 0.32	46.97 ± 4.97*	39.00 ± 2.44*	48.76 ± 2.31
BPF 100 ng/ml	7.59 ± 1.04	7.86 ± 0.71	14.71 ± 0.85	46.91 ± 4.53*	41.40 ± 1.89**	47.14 ± 3.23

Values are expressed as mean ± SEM. *, **, ***Significant difference at probability value $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared to control, respectively. ANOVA followed by Dunnett's comparison test. *BPF* Bisphenol F

Table 2 In vivo effect of subchronic Bisphenol F (BPF) on the different parameters

Parameter	Treatments ($n = 7$/group)					
	Control	BPF 1 mg/kg	BPF 5 mg/kg	BPF 25 mg/kg	BPF 50 mg/kg	BPF 100 mg/kg
Body weight gain (g)	35.00 ± 4.33	26.90 ± 5.23	25.10 ± 3.21	25.00 ± 4.33	29.20 ± 5.12	27.00 ± 4.33
Right Testis weight (g)	1.04 ± 0.05	1.18 ± 0.03	1.02 ± 0.03	1.14 ± 0.06	1.22 ± 0.05	1.12 ± 0.07
Left testis weight (g)	1.16 ± 0.02	1.16 ± 0.04	1.12 ± 0.02	1.14 ± 0.05	1.16 ± 0.03	1.12 ± 0.05
SOD (u/mg protein)	45.14 ± 1.19	34.29 ± 3.75	34.38 ± 1.48	36.26 ± 5.03	31.97 ± 4.63	32.54 ± 3.38
POD (nmole)	16.55 ± 0.43	15.18 ± 0.67	13.70 ± 1.12*	12.35 ± 0.39**	13.15 ± 0.64**	13.71 ± 0.68*
CAT (u/mg Protein)	16.08 ± 0.73	14.12 ± 1.01	13.03 ± 0.57*	12.99 ± 0.70*	12.91 ± 1.01*	11.59 ± 0.59**
LPO (min/mg Tissue)	13.13 ± 0.73	12.02 ± 0.80	13.35 ± 0.32	14.93 ± 0.58	15.35 ± 0.38*	15.62 ± 0.50*
Total ROS (U/g tissue)	0.94 ± 0.20	0.89 ± 0.05	0.96 ± 0.20	2.29 ± 0.42	2.78 ± 0.47*	3.11 ± 0.61**
Protein (mg/0.5 g)	341.91 ± 6.45	287.90 ± 21.72	280.23 ± 6.62*	278.90 ± 11.16*	274.77 ± 7.90*	267.81 ± 12.44**

Values are expressed as mean ± SEM. *, **, ***Significant difference at probability value P < 0.05, P < 0.01, and P < 0.001 compared to control, respectively. ANOVA followed by Dunnett's comparison test. *SOD* superoxide dismutase, *POD* peroxidase, *CAT* catalase, *LPO* lipid peroxidation, *ROS* reactive oxygen species

stress marker was determined in the reproductive tissues. A significant ($P < 0.05$) increase in the LPO content was observed in BPF 50 mg/kg and BPF 100 mg/kg treated groups when compared to control. However, the other doses of BPF did not show a significant effect as compared to control.

Similarly, a significant increase in ROS level was observed in BPF 50 mg/kg ($P < 0.05$) when compared to control. Total ROS was increased significantly ($P < 0.001$) in BPF 100 mg/kg as compared to control. However, total ROS was not altered by BPF 1, 5, and 25 mg/kg groups when compared to the control.

Total protein in the testis showed a significant reduction in BPF 5 mg/kg ($P < 0.05$), BPF 25 mg/kg ($P < 0.05$), and BPF 50 mg/kg ($P < 0.05$) as compared to the control. On the other hand, BPF 100 mg/kg treatment group showed a significant reduction ($P < 0.01$) in protein levels as compared to control.

Bisphenol F effects on the different hormones of male rats administrated with different concentrations for 28 days

Plasma testosterone, LH, FSH, and intra-testicular testosterone in the BPF different treated groups and control is presented in Table 3. Testosterone concentration was reduced significantly ($P < 0.05$) in BPF 25 mg/kg and 50 mg/kg treated groups. Similarly, BPF treatment caused a significant reduction ($P < 0.01$) at a dose level of 100 mg/kg. However, BPF in 1 and 5 mg/kg treated groups did not affect testosterone concentrations significantly.

Plasma LH concentrations reduced significantly in BPF 25 and 50 mg/kg ($P < 0.05$) as compared to the control. A significant reduction ($P < 0.01$) was also observed in BPF 100 mg/kg when compared to the control. On the other hand, BPF 1 and 5 mg/kg doses did not reduce plasma LH concentrations as compared to the control.

FSH reduced significantly in BPF 25 mg/kg ($P < 0.01$) as compared to the control. A significant reduction ($P < 0.001$) was also observed in BPF 50 and 100 mg/kg when compared to the control. On the other hand, BPF 1 and 5 mg/kg treatment did not reduce plasma FSH concentrations as compared to the control.

Intra-testicular testosterone in the testis after 28 days of exposure showed a significant reduction in BPF 25, 50, and 100 mg/kg ($P < 0.01$, $P < 0.001$, and $P < 0.01$, respectively) as compared to the control. Intra-testicular testosterone was not different in BPF 1 and 5 mg/kg treated groups than control (Table 3).

Table 3 Subchronic effect of Bisphenol F (BPF) on the intra-testicular testosterone, plasma testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) production in rats

Groups	Parameters			
($n = 7$/group)	Plasm testosterone (ng/ml)	Intra-testicular testosterone (ng/g tissue)	LH (ng/ml)	FSH (IU/ml)
Control	6.03 ± 0.35	55.32 ± 1.14	1.71 ± 0.07	0.92 ± 0.01
BPF 1 mg/kg	4.48 ± 0.50	52.44 ± 2.71	1.62 ± 0.09	0.83 ± 0.03
BPF 5 mg/kg	4.34 ± 0.51	51.48 ± 2.01	1.52 ± 0.05	0.76 ± 0.10
BPF 25 mg/kg	3.99 ± 0.64 *	46.71 ± 1.87 **	1.43 ± 0.05*	0.63 ± 0.02**
BPF 50 mg/kg	3.69 ± 0.53 *	44.16 ± 1.14 ***	1.39 ± 0.07*	0.59 ± 0.02***
BPF 100 mg/kg	3.20 ± 0.25 **	45.34 ± 1.04 **	1.20 ± 0.04***	0.44 ± 0.03***

Values are expressed as mean ± SEM. *, **, ***Significant difference at probability value $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared to control, respectively. ANOVA followed by Dunnett's comparison test

Morphological changes in testes and epididymis after exposure to bisphenol F (BPF)

Effect of BPF exposure on the seminiferous tubule area, interstitium area, seminiferous tubules diameter, and epithelial height in testicular tissue are presented in Table 4 and Fig. 1. There was no significant difference observed in the (%) area of seminiferous tubule and (%) area of interstitium of different treatment groups of BPF as compared to control. Similarly, a non-significant difference was observed in the diameter of seminiferous tubules in all treated groups as compared to control. There was significant ($P < 0.0.5$) reduction in epithelial height in BPF 50 mg/kg and 100 mg/kg groups when compared to the control. On the other hand, epithelial height was not different in BPF 1, 5, and 25 mg/kg treated groups than the control.

Transverse sections of testicular tissues of the control group were observed with thick epithelium, sperm-filled lumen, and seminiferous tubules (Fig. 1). Seminiferous tubule arrangement and shape was not very different in all treated groups when compared to the control. Though the pattern of epithelium was thin and the number of secondary spermatocytes was reduced in the treated groups when compared to the control. However, the groups with the higher doses of BPF were observed with few tubules and there were very few elongated spermatids in the lumen when these groups were compared with the control (Fig. 1).

Morphometry of different parameters of caput and cauda epididymis region after different BPA exposures did not show any significant difference in any of the parameter (tubular and lumen diameter, epithelial height, and percentage of epithelium and lumen) as compared to the control presented in Table 4 and Fig. 2. The shape of cauda and caput of the epididymis in the control was not very different from that of the treated groups. In the groups treated with 25, 50, and 100 mg/kg/day there were few empty lumens observed in each epididymis section when compared to the control though there was no loss of stereocilia observed.

The number of different cell types in the seminiferous tubules presented in Fig. 3. A significant difference was not observed in any of the treated group with different concentrations of BPF as compared to the control. Though the number of cells like spermatids and spermatocytes had decreased in some of the treated groups when compared to the control, the reduction was not statistically different when the comparison was done with the control.

Discussion

Although numerous studies have been published on the effects of BPA on the reproductive functions of male rats, the underlying mechanisms remain unclear. BPA displays non-monotonic dose-response functions [26]. Current knowledge on the biological and potential toxicological effects of BPA analog, especially on the reproductive system, is limited. The main purpose of the current study was to understand how safe is the BPA analog "BPF" from the medical point of view using rat models. Increased exposure of BPA during the prepubertal and pubertal period may affect the normal development and functions of reproductive organs, and the

Table 4 Oral subchronically administered rats with Bisphenol F (BPF) testis, caput, and cauda epididymis morphometry after 28 days of exposure

Parameter		Treatments ($n = 7$/group)					
		Control	BPF 1 mg/kg	BPF 5 mg/kg	BPF 25 mg/kg	BPF 50 mg/kg	BPF 100 mg/kg
Testis	Area of seminiferous tubule (%)	90.02 ± 0.98	88.85 ± 2.18	88.87 ± 2.34	87.37 ± 1.34	86.73 ± 1.41	85.65 ± 2.56
	Area of Interstitium (%)	19.02 ± 0.79	18.20 ± 0.88	17.43 ± 0.37	17.27 ± 0.54	16.88 ± 0.41	15.90 ± 1.50
	Seminiferous tubule diameter (μm)	213.91 ± 2.51	211.08 ± 5.49	209.09 ± 2.81	208.98 ± 0.72	207.48 ± 0.84	207.47 ± 1.48
	Epithelial height	77.27 ± 1.94	74.47 ± 1.95	73.71 ± 3.02	72.38 ± 1.40	69.16 ± 1.30*	68.13 ± 2.07*
Caput	Tubular diameter (μm)	403.40 ± 6.76	399.60 ± 4.28	398.20 ± 3.78	396.20 ± 3.94	394.80 ± 4.61	396.40 ± 2.97
	Lumen diameter (μm)	300.00 ± 7.05	298.60 ± 7.50	295.00 ± 4.14	297.20 ± 4.57	292.60 ± 4.82	290.60 ± 4.34
	Epithelial height (μm)	31.40 ± 1.80	29.40 ± 3.84	28.00 ± 1.41	28.20 ± 3.10	27.00 ± 0.83	26.60 ± 1.16
	Epithelium (% age)	37.95 ± 1.99	34.20 ± 1.01	33.60 ± 0.81	32.80 ± 1.49	32.40 ± 2.59	32.00 ± 0.63
	Lumen (% age)	70.85 ± 1.65	69.00 ± 3.21	67.86 ± 1.78	67.25 ± 0.91	68.34 ± 2.40	66.25 ± 7.74
Cauda	Tubular diameter (μm)	482.80 ± 4.58	478.60 ± 3.60	476.60 ± 5.26	477.80 ± 4.03	475.80 ± 6.51	474.80 ± 4.03
	Lumen diameter (μm)	432.60 ± 2.98	436.80 ± 4.68	435.20 ± 4.65	441.00 ± 4.32	438.80 ± 0.96	439.20 ± 3.36
	Epithelial height (μm)	33.25 ± 2.32	34.50 ± 1.79	35.75 ± 0.49	35.80 ± 4.03	36.20 ± 2.07	38.40 ± 3.40
	Epithelium (% age)	39.00 ± 1.54	43.75 ± 1.89	44.75 ± 2.10	42.75 ± 2.41	44.10 ± 1.73	45.00 ± 1.18
	Lumen (% age)	63.25 ± 1.45	60.00 ± 2.87	62.25 ± 1.73	60.50 ± 2.77	59.25 ± 3.04	58.65 ± 2.47

Values are expressed as mean ± SEM. *Significant difference at probability value $P < 0.05$ compared to control. ANOVA followed by Dunnett's comparison test

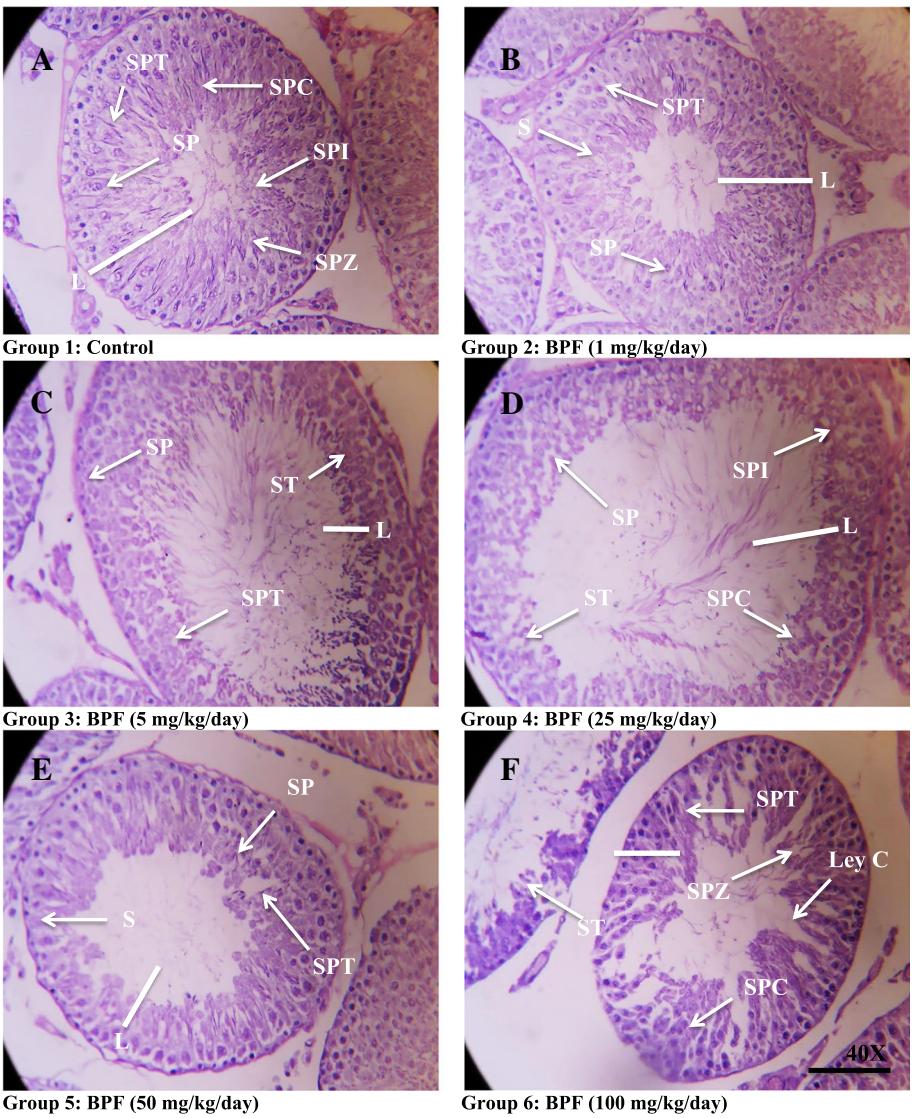

Fig. 1 Photomicrographs of rats testicular tissues of control and treated animals with different concentrations of BPF. The control (**a**) reveals normal germ cells: spermatogonia (SP), spermatocytes (SPC), spermatids (SPT), spermatozoa (SPZ). **b**–**f** Treated groups with BPF (1, 5, 25, 50, and100 mg/kg/day) showing changes in the testicular tissues seminiferous tubules with epithelium (Line without arrowhead), showing change in the testicular parenchyma, absence of sperm in lumen, seminiferous tubules with germ cells, Leydig cells (LeyC), absence of sperm in lumen of tubules and spermatids. Presenting ST, seminiferous tubules; SP, spermatogonia; SPC, spermatocytes; SPT, spermatids; SPZ, spermatozoa; IT, interstitial tissue; LeyC, Leydig cell (White arrow). H&E (× 40)

resulting toxic effects of these chemicals may affect the regulatory genes involved in the development of follicles in females and sperms in males. The consumption of BPA alternatives is at rising due to strict regulations on the use of BPA in some countries [27, 28]. A study suggested a possible association between BPA levels and increased risk of prosocial behavior and between MECPP levels and increased risk of conduct problems [29]. The structural similarity of BPF with BPA marks it as an endocrine disruptor and in vitro data has also revealed that BPF has a binding affinity with receptors which change the testosterone secretions in the fetal testis and can also induce cell proliferation. In vivo studies have shown that BPF influences the expression of sex hormone-regulated genes and also has developmental and reproductive effects in mammals. Recently published data regarding some of the BPA analogs has upturned concerns that whether the so-called safer analogs of BPA are more alarming to both human and wildlife [30]. In the present study, we conducted both in vivo and in vitro studies to evaluate the effects of BPF on the reproductive functions of male rats.

In the in vitro study, we incubated testicular tissues with different concentrations of BPF for 2 hours. The

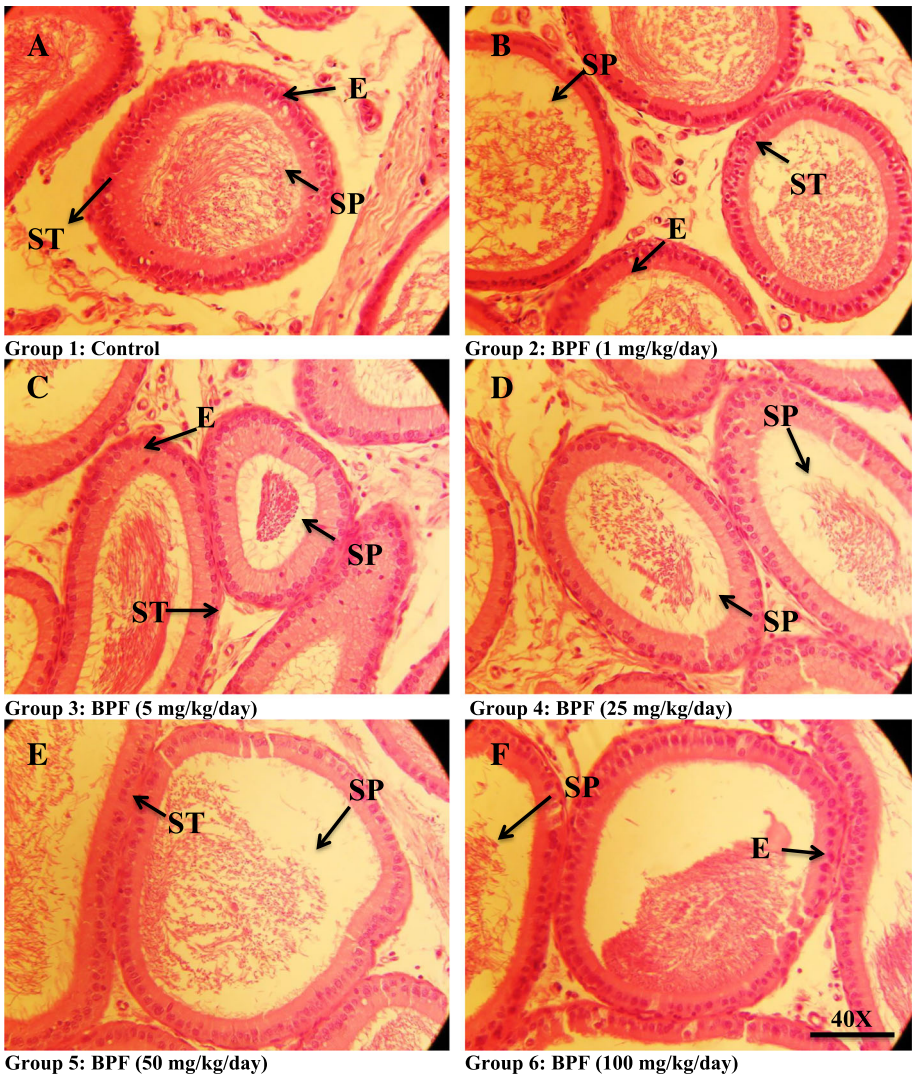

Fig. 2 Photomicrograph of caput epididymis tissue showing **a** control; with compact arrangement of caput tubules with sperm-filled lumen **b** BPF (1 mg/kg/day)-exposed group, presenting normal caput tubules like in the control. **c** BPF (5 mg/kg/day) exposed group showing seminiferous tubules with less number of sperm in the lumen (arrow). **d** BPF (25 mg/kg/day)-exposed group presenting caput tubules with empty lumen (arrow). Similarly, **e** BPF (50 mg/kg/day)-exposed group showing less number of sperms in the lumen. **f** BPF (100 mg/kg/day)-exposed group showing less number of sperms and empty lumen (arrow). Presenting SP, spermatozoa; ST, seminiferous tubules; E, epithelium. H&E (× 40)

incubated tissues did not show any significant change on the antioxidant enzymes as CAT, SOD, and POD. BPA and its analogs have a toxic influence on the formation of ROS inside the body, and studies have also shown that these phenols not only increase in the levels of ROS but also lead into oxidative stress inside many cellular networks [31]. Similar to data reported in BPS, we observed increase ROS and lipid peroxidation in testicular tissues after exposure to BPF in vitro [16, 32]. The results of the in vivo study showed dose-dependent effects of BPF on the oxidative stress in the reproductive system of male rats. The groups exposed to higher concentrations of BPF showed a significant difference in the histology of the reproductive tissues by reducing the sperm number in the epididymis and decreasing the height of epithelial tissues. Androgens also play an important role in the normal development of the male reproductive system [26, 33, 34]. BPF higher exposure groups were observed with an elevated level of testosterone which also leads to higher oxidative stress as compared to the low-dose-exposure groups.

Exposure to BPF caused induction of ROS which lead into an increase in the levels of LPO and activation of antioxidant enzymes which are in line with the earlier studies where BPA exposure degraded protein and altered antioxidant enzymes [35]. In the in vivo study, BPF exposure also increased the levels of LPO and also altered the levels of SOD and CAT which also indicated

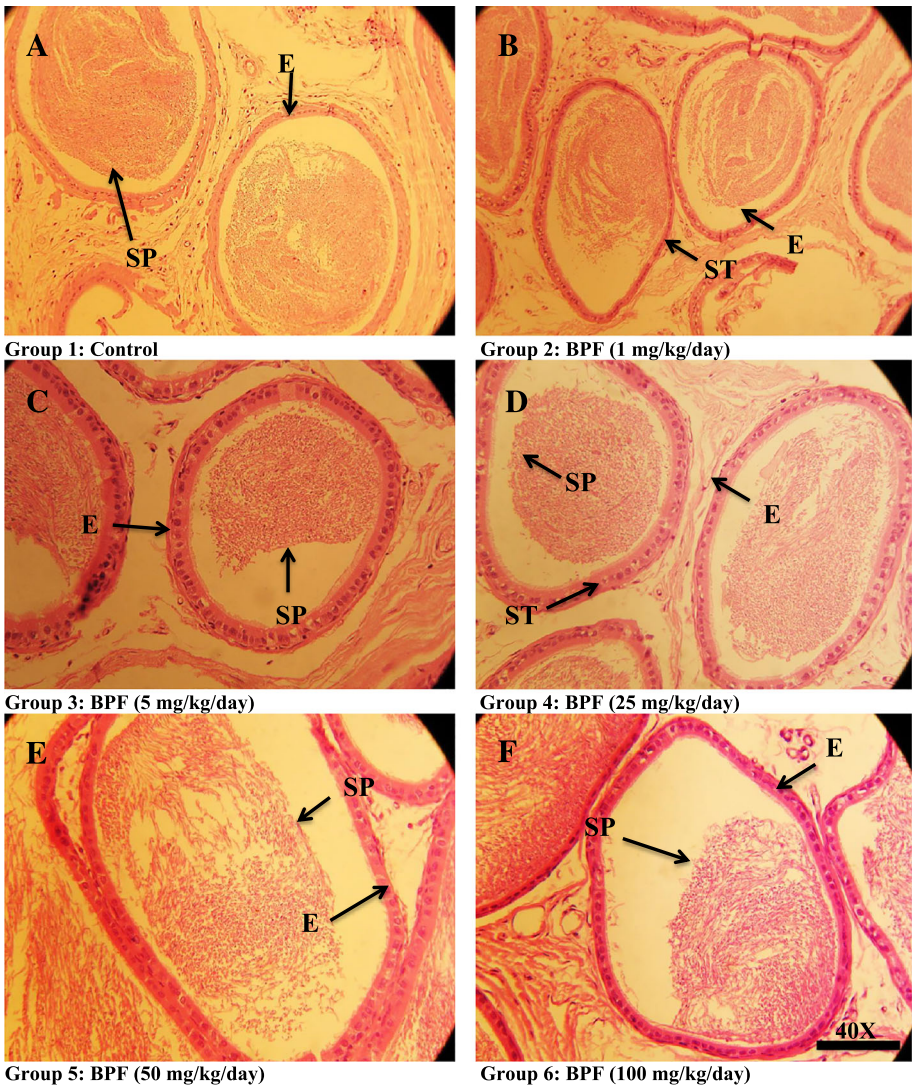

Fig. 3 Photomicrograph of cauda epididymis tissue showing **a** control; with compact arrangement of cauda tubules with sperm-filled lumen. **b** BPF (1 mg/kg/day)-exposed group, presenting normal caput tubules like in the control. **c** BPF (5 mg/kg/day)-exposed group, presenting cauda tubules with sperm-filled lumen. **d** BPF (25 mg/kg/day-exposed group presenting cauda tubules with less sperm in the lumen. Similarly, **e** BPF (50 mg/kg/day)-exposed group presenting cauda tubules with fewer sperm in the lumen. Likewise, **f** BPF (100 mg/kg/day)-exposed group presenting cauda tubules with empty spaces and fewer number of sperm in the lumen. Presenting SP, spermatozoa, ST, seminiferous tubules; E, epithelium. H&E (× 40)

oxidative stress. This change occurred because of oxidative stress in the reproductive tissues caused by exposure of BPF different concentrations which also reduced the levels of proteins and antioxidant enzymes which are similar to the findings of some previous studies [36–39].

We observed the substantial change in both plasma and intra-testicular testosterone in the in vivo study. Our results are in accordance with previous researches which indicated altered levels of different hormones after exposure to BPA and some of its analogs [19, 34].

The process of Spermatogenesis is controlled by different reproductive hormones and cellular interactions inside the testes. ROS and disturbed antioxidant enzymes lead to disturbed spermatogenesis [40]. In the testicular tissues, we observed a reduction in the number of spermatids, alerted epithelial height and seminiferous tubules, and reduced concentrations of testosterone. Some previous studies are in accordance with our current study on the exposure of BPF where exposure to BPA and some of its analogs altered steroidogenesis and lead into oxidative stress in the different tissues [19, 34]. Similarly, our current study results also showed that BPF not only alters spermatogenesis in the testis but also causes a reduction in the levels of testosterone secretions. Further studies are required both in vitro and in vivo which can show the molecular and cellular mechanisms of these BPA

analogs specific response in the environmental hazard assessment which will let us better understand the mechanisms through which BPA analogs endocrine disruption on different tissues be analyzed.

Conclusions

The results of our present study showed that BPF at higher dose exposures may possibly have outcomes in oxidative stress and disturbed reproductive hormones. Thus, the use of BPA analogs should be carried out with caution, especially until the effective risk assessment is conducted. Further studies need to analyze the molecular basis of these alterations both in vivo and in vitro studies which will let us understand how BPF can still have an effect on the physiology of different tissues inside the body.

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Authors' contributions

AU and SJ designed the study, conceived the study, and analyzed the results. AU conceived an initial part of the study, performed the experiment, and helped in compiling the results. SR helped in writing the results. SJ, SR, MP, and TA wrote the paper with input from all other authors. AU, SR, TA, MP, and AA made a substantial contribution in the interpretation of data and revising the manuscript for intellectual content. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable

Ethics approval and consent to participate

This study makes use of animals and the experimental protocol was approved (BAS#0256) by the ethical board of Quaid-i-Azam University, Islamabad, Pakistan.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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