A Physiologically based Approach to Access the Hazardous Effects of Carcinogen Food Contaminant Furan as an Endocrine Disruptor on Reproduction

By

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FACULTY OF BIOLOGICAL SCIENCES

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A thesis submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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2020

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled "A Physiologically Based Approach to Access the Hazardous Effects of Carcinogen Food Contaminant Furan as an Endocrine Disruptor on Reproduction" was conducted by **Ms. Humaira Rehman Jarral** under the supervision of **Prof. Dr. Sarwat Jahan**. No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Zoology of Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Field of Reproductive Physiology.

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" IN THE NAME OF ALLAH, THE MOST MERCIFUL, THE MOST BENEFICENT AND THE MOST COMPASSIONATE "

Dedication

"I dedicate my humble effort to my mentors ***Dr. Sarwat Jahan & Dr. Imdad Ullah Salaar*** *for their countless support, affections and encouragement during my journey and they make me able to get such success and honour in life"*

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LIST OF ABBREVIATIONS

Abbreviations	Full Names
C	Cortisol
cm	Centimeter
dd	Double distilled water
DHBA	2,3-Dihydroxybenzoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DA	Dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
DSP	Daily sperm production
EDCs	Endocrine Disrupting Chemicals
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
EOGRTs	Extended One Generation Reproductive Toxicity study
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FSH	Follicle-stimulating hormone
g	Gram
GH	Growth Hormone
GSH	Reduced Glutathione Activity
GST	Glutathione-S-Transferase Activity
hr	Hour
HVA	Homovanilic acid
IARC	International Agency for Research on Cancer
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LPO	Lipid Peroxidase
mgL^{-1}	Milligram per litre
min	Minute

mL	Millilitre
mm	Millimeter
mmole	Millimole
mmole/L	Millimole per litre
Ms222	Ethyl 3-aminobenzoate methane sulfonic acid
ng/L	Nanogram per litre
ngg^{-1}	Nanogram per gram
NTP	National Toxicology Programme
NE	Norepinephrine
OECD	Organization for Economic Co-operation and Development
PBS	Phosphate Buffer Saline
PH	Potential hydrogen
PND	Postnatal day
PNMC	3-methyl-4-nitrophenol
POD	Peroxidase
ppm	Part per million
RMPA	Regular melting point agarose
rpm	Rounds per minute
SCGE	Single-cell gel electrophoresis
Sec	Second
SOD	Superoxide Dismutase
T	Testosterone
TBARS	Thiobarbituric acid reactive substances
TMB	3,3,5,5-Tetramethylbenzidine
Triton X-100	Dithiothreitol
5HIAA	5-Hydroxyindoleacetic acid
5HT	5-hydroxytryptamine

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GENERAL ABSTRACT

Various chemicals or toxicants have been classified as endocrine-disrupting chemicals (EDCs) because of their ability to alter animal pathophysiology. EDCs exogenously enters the body and make alterations in the normal functioning of endocrine systems and also caused adverse health effects in an organism or its offspring. EDCs target every endocrine axis, their action is not limited to some single-axis or organ, are hypothalamus-pituitary -thyroid (HPT), hypothalamus-pituitary-gonads (HPG), hypothalamus-pituitary-adrenal (HPA) axis and associated organ systems are the main targets of EDCs. These chemicals may also affect the central nervous system (CNS) and hypothalamic and pituitary functions. Many harmful compounds or EDCs are produced during food processing including furan and acrylamide. Furan (C4H4O) is a colourless volatile liquid, which is used in several industries and produced in a variety of food items during heating processes such as coffee, sauces, soups, canned, jarred foods, infant formulae, and baby foods, exerting adverse effect to human health including reproductive health. It is also present in the environment as the main constituent of cigarette smoke, wood smoke, and exhaust gases from engines. The Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) described the carcinogenic and genotoxic mechanism of furan. Previous studies reported that furan caused various types of adverse carcinogenic effects on the biological system of humans and animals. Furan caused significant changes in histological and DNA structure of ovarian cells, malondialdehyde levels, and antioxidant enzyme activities. An *in vitro* study showed that furan incorporates chromosomal aberrations and sister chromatid exchanges (SCEs) in Chinese hamster ovary (CHO) cells and Chinese hamster V79-derived cell line. The previous investigation showed that furan has antiandrogenic activity. In a human study, furan exposure generated reactive oxygen species and affected the lymphocytes and sperm cells. However, data regarding the adverse effect of furan on the mammalian reproductive system is still scant.

Objectives: Present study was designed for a comprehensive assessment of the food-based toxicant furan on the mammalian reproductive system. The main objectives of the study are:

- To investigate the toxicological and endocrine disrupting potentials of furan in rats, an *in vitro* approach was used to target the process of androgenesis and oxidative stress in testis and epididymal sperms.
- To determine the possible *in vivo* toxic effects of furan experience on the reproductive system of the male through 28 days of study.
- To examine the effects of furan on sperm production, maturation, and functions to regulate or inhibit specific targets that are involved in infertility.
- To evaluate the effect of furan on the development of gonads, exposed during the neonatal period.
- To study effects of furan exposure on HPG and HPA-axis by determining plasma T, LH, FSH, cortisol concentrations, and the brain monoaminergic activity i.e., 5HIAA/5HT, DOPAC/DA, HVA /DA ratios, NE level.
- To establish a link between furan and endocrine disruption, a bivariant correlation between T, LH, FSH concentrations, brain monoaminergic activity, 5HIAA/5HT, DOPAC/DA, HVA/DA ratios, NE level, and cortisol were performed with the aim of the effect of stress on reproduction.
- To find out the potential toxic effect of furan on reproductive performance of rats in F0 (parental) and F1 (offspring). A frequent oral dose based extended one-generation reproductive toxicity study was performed.

Materials and Methods; Stock solution of furan (Cat#185922, 99% purity) was prepared in corn oil. Testis tissues and sperm of male adult rats were properly incubated with artificial media having various concentrations of furan (0, 1, 100, 500, and 1000 $\mu g\ mL^{-1}$) for 2 hrs at 37 °C with 5 % CO_2 and 95 % air. The different concentrations of Furan were dissolved in dimethyl sulfoxide (DMSO) and further dilution was made through media of cell culture. The overall concentration of DMSO in the cell culture media was less than 1 per cent. Oxidative stress in the testicular sperm and tissues were concluded through antioxidant enzyme activity. Testicular testosterone (T) concentration was determined through the ELISA technique. Whereas the DNA damage was measured through comet assay procedure in sperm cells. To achieve the second objective, based on *in vitro* study an *in vivo* study was performed. In this part of the experiment different doses (5-40 mg kg^{-1}) of furan were used through an oral route for 28 days. For hormones analysis blood was collected. Brain tissues were used for the analysis of monoamine, while for sperm analysis, histopathology, and oxidative

stress determination testicular and epididymis tissues were used. In the third part of the experiment, a neonatal exposure study was performed during the gonads' development period. In this study, the male rat pups were subcutaneously injected with various concentrations of furan (1 - 20 mg kg^{-1} furan in 50 µL corn oil) after PND 4 to PND 15 and were kept under observation till adulthood to inspect any amendments in the reproductive systems. Different parameters were monitored and compared with the control group. One-generation reproduction toxicity study was performed. In this study parents F0 (for male rats 70 days and female rats 14 days) were exposed to furan at a concentration of 1, 2.5, 5, and 10 mg kg^{-1} furan dissolved in 50 µL corn oil respectively before mating. Male rat's dose exposure continued during mating whereas female rats received doses of furan during pregnancy and lactation. Complete monitoring of male rats (pre breed and during mating periods) and female rats during (pre breed, gestation, and lactation period) for complete physical examination. The number of living and dead pups, their weights, sex ratio, anogenital distance, nipple retention, and survival rate, etc were recorded during physical examination. Monitor all the reproductive parameters of F1 generation animals. At the age of 10 weeks, the F1 rats were properly marked for different reproductive examinations (body weights, hormonal profile, and histopathology).

Results; In the *in vitro* study after exposing the testicular tissues and epididymal sperms to furan, oxidative stress was increased along with a decrease in antioxidant activity. At the maximum concentration, furan tempted the formation of reactive oxygen species and increased DNA fragmentation in the rat sperm cells. Also, furan cause reduced testosterone (T) secretion by the testes. The sub-chronic study of Lipid Peroxidation (LPO) and Reactive Oxygen Species (ROS) were significantly increased in the higher doses treated groups, whereas the activity of the antioxidant enzyme was reduced significantly in testicular tissues. The sub-chronic furan further decreased the plasma and intra-testicular T, LH, and FSH although, the level of cortisol increased in the groups treated with higher doses of furan. Brain monoaminergic activity was also interrupted in higher dose treatment groups. A significantly elevated oxidative stress was observed in testes while sperm parameters were reduced. Testicular and epididymis morphology results also revealed significant alterations after treatment with higher concentrations of furan. However, not a highly significant delay in puberty onset was reported in the groups treated with higher doses. Plasma hormonal analysis

demonstrated a significant reduction in T, LH, FSH, GH, and a significantly elevated level of cortisol was observed in the high dose groups as compared to the control. Brain monoaminergic activity was also interrupted by exposure to furan. Histopathological variations involved a drop in epithelial height and diameter of the seminiferous tubules. Sperm parameters were affected by the high dose treated group than the control. However, F1, an extended one-generation reproductive toxicity study was accomplished to observe the adverse effects of furan in the next generation. After exposure, we observed significant changes in the weight of parental F0 animals. We found no significant change in the Parental F0 reproductive parameters. We also observed F1 litters parameters during the lactation period. At the pubertal stage, we observe a decline DSP in F1 generation male rats. Plasma hormonal concentration of testosterone (T) and Luteinizing hormone (LH) was decreased in F1 males respectively. Some histopathological changes were also observed in the F1 generation, whose parents were previously exposed to the high dose of furan.

Conclusion; It is concluded that furan exposure caused reproductive toxicity by generating ROS in *in vitro* and *in vivo* studies. Besides its effect in adult animals, exposure during the neonatal stage of development we find clear evidence that exposure to the two highest doses broadly and consistently affects the development of the male reproductive system, which ultimately leads to a reduction in fertility rate. Furan can cause organizational effects on the development of male reproductive systems. This study provides important preclinical data on the minimal dose of furan at which endocrine disruption can occur. Otherwise in F1, extended generation the reproductive toxicity investigation a prominent change in sex hormone concentrations was evident, and slight histological alterations were also seen in testis. These findings suggest that furan has detrimental effects on the developmental stages of life because the developmental period is the most sensitive period of life. Due to these findings, it is concluded here that the use of furan may be a matter of high concern for human life and health. The toxicological profile of furan presented in these studies supports the identification of furan as an endocrine disruptor.

GENERAL INTRODUCTION

In recent decades, there has been great concern about the adverse effects of different environmental toxicants on human health and reproduction. Environmental factors such as exposure to environmental toxicants are one of the major causes of reproductive dysfunctions in humans. Various environmental chemicals/toxicants have been classified as endocrine-disrupting chemicals (EDCs) because of their ability to alter animal pathophysiology. It is stated as, any substance or mixture that exogenously enters in the body and make alterations in the functions of endocrine systems and also caused adverse health problems in a whole organism or its offspring or the whole population is called EDCs (WHO/IPCS, 2002). More than 800 chemicals are known as EDC due to their endocrine disruption nature (EHP, 2013). EDCs are constituted a long list of chemicals including dioxins, polychlorinated biphenyls (PCBs), and insecticide, pesticides, herbicides, alkylphenols, fungicides, phthalates, phytoestrogens, bisphenol A, polybrominated biphenyls, pharmaceutical drugs, food-based acrylamide, furan, heavy metals like lead, cadmium, arsenic, and mercury (Crisp *et al.*, 1998; Sharara *et al.*, 1998; Hodges *et al.*, 2000; Dickerson and Gore, 2007; Robin and Clanci, 2007; De Coster and Van Larebeke, 2012; Gnessi, 2013; Pandir, 2015; Lehmann *et al.*, 2018; FDA, 2004). The mechanism of action of EDCs is depending on their binding capability with endocrine nuclear receptors (NRs) (De Coster and Van Larebeke, 2012; Gore *et al.*, 2015).

EDCs can activate several hormone receptors like estrogen, androgen, estrogen-related receptor, pregnane X, constitutive androstane, aryl hydrocarbon, thyroid hormone, retinoid X, and glucocorticoid receptor as well as mimic the natural mechanism of hormones (Mnif et al., 2011; Schug *et al.*, 2011). EDCs can interrupt synthesis, transportation, metabolism, exclusion of endogenous hormones (De Coster and Van Larebeke, 2012; Gore *et al.*, 2015). Additionally, EDCs disturbed the living endocrine mechanisms with addictive or synergistic effects. EDCs in relationship with other EDCs may result in a more adverse cocktail effect (Nohynek *et al.*, 2013; Balaguer *et al.*, 2017). EDCs target every endocrine axis, their action is broader than hypothalamus-pituitary gland-adrenal (HPA), hypothalamus-pituitary gland-thyroid (HPT), and hypothalamus-pituitary gland-gonads (HPG), being the crucial goals of EDCs (Gore *et al.*, 2015; Kabir *et al.*, 2015). The function of the central nervous system

(CNS), hypothalamic, and pituitary is damaged by EDCs (De Coster and Van Larebeke, 2012; Gore *et al.*, 2015; Kabir *et al.*, 2015). For human life HPA and HPG axis, proper functioning is very important. Interaction of EDCs with HPA and HPG axes alters proper functioning. Developmental stages are also vulnerable since changes can become permanent when animals are exposed to EDCs during the organ development stage (Richter *et al.*, 2007) changing the hormonal profile irreversibly (Lafuente *et al.*, 2003; Hinson and Raven, 2006; Liu *et al.*, 2010; Cooke *et al.*, 2014; Sajjad *et al.*, 2018). Irreversible and long-lasting changes are in terms of the organizational effects of EDCs (Young, 1964; Arnold and Breedlove, 1985). Exposure to EDCs during the gestational and neonatal periods resulted in permanent alteration in the mechanism of hormone actions and bring changes in the development of related structures (Csaba, 1980; Makri *et al.*, 2004). EDCs may exhibit through different ways contaminate the human body (Lauretta *et al.*, 2019). Normally, the intake of food, inhalation, and contact are the common pathways of EDCs exposure (De Coster and Van Larebeke, 2012; Gore *et al.*, 2015; Kabir *et al.*, 2015).

In animals and humans, various reports showing the specific effects of EDCs at different developmental periods of life. According to the executive order (13045; 1997), issued by federal agencies in the United States, identification and assessment of environmental health risks affecting children's health must be highly prioritized (EPA, 2017). Recent literature reported that EDCs exposure through a developmental period not only damages the living organism but also affects future generations (Lauretta *et al.*, 2019).

Heat-induced food toxicants

The heating process of foods provides many tempting sensorial qualities, including taste, flavour, and colour. However, some toxic compounds are formed during the heating process which includes furan and acrylamide may constitute increased health hazardous for consumers. Heat-induced food-based toxicants have attracted widespread attention in the current era i.e., furan, acrylamide, and chloropropanols (Wenzl *et al.*, 2007). The occurrence of chloropropanols and furan in food was first reported in the 1970s, while acrylamide was identified in 2002 (Velíšek *et al.*, 1978; Swedish National Food Administration 2002). International

Agency for Research on Cancer declare acrylamide and furan as a carcinogen to humans (IARC, 1995). In food, the presence of furan and acrylamide is a great issue and these compounds are listed in every FDA Center for Food Safety and Applied Nutrition (CFSAN) program. Later on, the presence of many environmental toxicants and heat-induced food toxicants including bisphenol, arsenic, chlorinated dioxins, TCDD, furan, polychlorinated biphenyls, DDT and acrylamide are presence has been reported in breast milk, infant formulas (Lehmann *et al.*, 2018) baby biscuits and follow on formulas (Mojska *et al.*, 2012) are reported. FDA has declared furan and acrylamide as food toxicants, having endocrine disrupting potentials (Robin and Clanci, 2007; FDA, 2004).

Furan

Furan (C4H4O; CAS-Nr. 110-00-9) is an organic compound with high volatile and lipophilic properties, acts as an important intermediate constituent in the preparation of several chemicals (NTP, 1993). Furan was initially reported in food items by (Maga and Katz, 1979). The IARC has declared furan as a potentially carcinogenic substance (IARC, 1995). European Food Safety Authority (EFSA) declared furan as having carcinogenic and genotoxic mechanisms (NTP, 1993; EFSA, 2004). The FDA published data on furan in May 2004, while the EFSA Scientific Panel on Contaminants in the Food Chain (CONTAM) published a preliminary report on furan in 2004, and 2009 (EFSA, 2009). They reported the existence of furan in various food items and products, i.e., canned, jarred, and baked food, coffee, and baby food. It has been also reported that furan is an environmental and food toxicant that is generally present in infant formulae, canned, and junk foods (Pluim *et al.*, 1993; Karacaoğlu and Selmanoğlu, 2010; Lehmann *et al.*, 2018).

Furan is also a by-product of solid foods and beverages exposed to high-energy radiation or temperature (Bolger, 2009; Bakhiya & Appel, 2010). Furan is also present in the environment as a component of wood smoke, cigarette smoke, exhaust gas from gasoline, and diesel engines (IARC, 1995). Ascorbic acid, amino acids, sugars, and fatty acids are the most important food ingredients that serve as precursors molecules for furan (Crews and Castle, 2007). Model chemical reactions with mixtures of precursors provide an important mechanism as to how furan is produced through

excessive heat processing. Previous observations revealed that dehydroascorbic acid, L-ascorbic acid, D-erythrose, and glycolaldehyde/L-alanine are an utmost vital precursor for furan (Perez Locas and Yaylayan, 2004).

Exposure to furan

Human food contains thousands of structurally different chemical substances, mostly from natural origin and components purposely added such as colourants, nutrients, and flavours. Moreover, maximal furan contents were found through treatments of heat e.g, baby food, meat products, cereals products, soups, and sauces (EFSA, 2009, 2010). In nutritional drinks, fruit/vegetable juices, and bakery products elevated concentrations of furan were reported (EFSA, 2010; FDA, 2004).

Exposure of humans to furan was assessed based on feeding and the amount of furan content in different food substances. Develop countries the average estimated exposure of adult humans to furan through food is 0.34 to 1.23 g/kg bw/day almost 0.78 g/kg bw/day (EFSA, 2009). At the age of 3–12 months mean estimated exposure is 0.27 to 1.01 g/kg bw/day (EFSA, 2009). In infants, 0.23–1.77 g/kg bw/day of furan was also reported (Minorczyk *et al.*, 2011). Coffee was also considered as the highest source of furan in adults (EFSA, 2009).In infants, the main source of toxicants furan is either breastfeeding or formula feeding (Lehmann *et al.*, 2018). Excessive levels of furan were reported in prune juices, baby carrot juices, and flavours of nutritional drinks (Wegener and López-Sánchez, 2010). The rye and wholegrain products presented excessive levels of furan (Wegener and López-Sánchez, 2010). Previous findings propose that bottled and canned drinks are substantial source of furan for infants (Wegener and López-Sánchez, 2010).

Toxicology of furan

Previous observations reported that furan caused various types of cancer and has an adverse effect on the biological system of humans and animals (IARC, 1995; Hamadeh *et al.*, 2004; Pandir, 2015; Uçar and Pandir, 2017). There is widespread exposure of humans to furan (Peterson *et al.*, 1993; Kim *et al.*, 2004; Moro *et al.*, 2012; Mariotti *et al.*, 2013). Numerous reports have revealed the adverse effects of furan on

the vital organs of rodents (Gill *et al.*, 2010; Selmanoğlu *et al.*, 2012; Webster *et al.*, 2014). Metabolites of furan are another source of toxicity (Kedderis, 1999). Furan induces hepatic necrosis and DNA damage, that closely linked with an elevation of serum alanine aminotransferase (ALT) in rats (Gill *et al.*, 2010; McDaniel *et al.*, 2012). Bas and Pandir (2016) described that furan exposure increases malondialdehyde (Rehman *et al.*) levels and decreases glutathione-S-transferase (Livingstone), superoxide dismutase, and catalase in rats. Thus far, limited information is available in the literature about the toxic mechanism of furan on the reproductive system of the male. Furan caused significant changes in histological structure, DNA structure of ovarian cells, malondialdehyde levels, and antioxidant enzyme activities (Uçar and Pandir, 2017). An *in vitro* study showed that furan brought sister chromatid exchanges (SCEs) and chromosomal abnormalities in Chinese hamster ovary and Chinese hamster V79-derived cell line (NTP, 1993; Glatt *et al.*, 2005). Some studies showed the antiandrogenic activity of furan (Karacaoğlu and Selmanoğlu, 2010; Cooke *et al.*, 2014). In a human study, furan exposure generated reactive oxygen species and affected the lymphocytes and sperm cells (Pandir, 2015). A clinical study reported that 11-week old babies had higher thyroid hormone levels when their mother's breastmilk contained higher than average dioxin and furan concentrations (Pluim *et al.*, 1993). Previously, reported that furan exposure via breastfeeding or food causes abnormalities in the fetus because such compounds can pass through the placental barrier (Van Wijnen *et al.*, 1990; Pluim *et al.*, 1993). In adult rats, exposure to furan resulted in decreased testosterone levels and sperm counts, impaired spermatogenesis and induced apoptosis in Leydig and germ cells (Karacaoğlu and Selmanoğlu, 2010; Cooke *et al.*, 2014).

Furan and apoptosis

Pandir *et al.* (2015) recorded apoptosis in an *in vitro* study of blood and sperm cells by furan induced apoptosis within the cells. In another *in vivo* study furan exposure caused apoptosis in germ cells (Karacaoğlu and Selmanoğlu, 2010).

Furan and oxidative stress

Reactive oxygen species (ROS) are endogenously produced and take part in various biological and chemical reactions (Moncada, 1991; Azzi *et al.*, 2004).

However, excessive production of ROS is deleterious when the antioxidant level is depleted (Embola *et al.*, 2002). The reproductive system of the human is susceptible to the toxicity of excessive production of ROS. Oxidative damage can lead to abnormal sperm morphology, along with decreased sperm motility, and egg penetration (Iwasaki and Gagnon, 1992; Sukcharoen *et al.*, 1996; Mazzilli *et al.*, 1994; Aitken and Curry, 2011). ROS production enhances lipid peroxidase that leads to alteration of acrosome reaction, sperm DNA damage, and fusogenic capacity (Ichikawa *et al.*, 1999; Zalata, 2004). Excessive ROS deteriorates the receptors of gonadotropin, lowers cyclic AMP, and reduces steroids secretion by affecting corpora leutea (Aten *et al.*, 1992). Spermatozoa are also affected by oxidative stress due to the presence of unsaturated fatty acids in the membrane (Aitken and Curry, 2011). Spermatozoa are equipped with mitochondria for the continuous supply of energy. Excessive ROS production disrupts the inner and outer mitochondrial membranes and releases cytochrome C, which activates apoptosis (Thuillier *et al.*, 2003). Living cells protect themselves through antioxidant enzymes that reduce ROS production as well as detoxify ROS through the conversion of hydrogen peroxidase (H_2O_2) and dismutation. The superoxidase dismutase (SOD) produces H_2O_2 through the process of dismutation while Catalase (CAT) converts H_2O_2 to water (Imlay *et al.*, 1988; Wiseman and Halliwell, 1996). In living physique contaminated elements usually target the liver because the breakdown of chemicals happened in the liver which ultimately leads to damaging hepatocytes and liver toxicity (Livingstone, 1991; Patel *et al.*, 2012). Due to low polarity furan can easily be passed through the biological membranes (Bakhiya and Appel, 2010) and quickly absorbed in the body (Crews and Castle, 2007).

Reported that exposure to this compound increases each day because of its formation in home overcooked food and baby products. Furan was also reported to occur in morning urine samples of humans in different concentrations (Ghosh *et al.*, 2015). Previous observations have suggested that the liver and kidney are the main focus of furan noxiousness. As the main portion of the defence mechanism of the living cell, CAT changes ROS to H_2O_2 and further convert H_2O_2 to water, that's water shield cells from ROS toxicity (Syed and Mukhtar, 2012). Furan was found to be toxic to the hepatocytes, pancreas, and renal cells. Furan exerts a toxic effect on the pancreas by exerting oxidative stress (El-Habiby *et al.*, 2017) if epigenetic alterations are closely linked to the effect of furan (Conti *et al.*, 2014).

Genotoxicology of furan

Furan caused significant changes in DNA structure, abnormalities in chromosomes, and exchanges of sister chromatids in the cells ovary and Chinese hamster V79 derived cell line (NTP, 1993; Glatt *et al.*, 2005). Furan is mutagenic to mouse lymphoma cells (McGregor *et al.*, 1988). Elevated doses of furan induced aberrations in the chromosome. Furan also causes ATP loss after bioactivation to metabolites that initiate a permanent hepatic mitochondrial oxidative phosphorylation and further activates endonucleases i.e., cytotoxic enzymes that break DNA double-strand before cell loss (Kedderis *et al.*, 1993; Kedderis, 1999). It has been reported, that 4 weeks of treatment of B6C3F1 mice splenocytes through gavage induced genotoxicity (Leopardi *et al.*, 2009). Hickling *et al.* (2010) reported that furan can indirectly damage liver DNA through the production of ROS (Hickling *et al.*, 2010).

Endocrine-disrupting potentials and reproductive toxicity of furan

The development of male reproductive system organs like prostate, Wolffian ducts, epididymis, seminal vesicles, and vas deferens depends upon the normal production of steroid hormones but EDCs interaction with these hormones badly affects their proper organs development and functioning (Sweeney et al., 2015). EDCs inhibit the 5α–reductase and aromatase enzymes, these enzymes are responsible for the conversion of the androgens to testosterone(Sweeney et al., 2015). Estrogen receptors ERα and Erβ expression levels are also disturbed by EDCs exposure. In humans, Erβ receptor expression is sustained through morphogenesis and cellular differentiation and is diminished in early puberty. Increased plasma androgens level also enhances the expression of the receptor that is linked with benign prostate hyperplasia and/or prostate cancer. Exposure to EDCs can affect the developmental stages of animals. Permanent changes occur when animals are exposed to EDCs during the stage of organ development (Richter *et al.*, 2007).

The exposure to EDCs also disturbs the hormonal profile (Lafuente *et al.*, 2003; Hinson and Raven, 2006; Liu *et al.*, 2010; Cooke *et al.*, 2014). Previously recognized by the FDA that food-based contaminants furan and acrylamide, having endocrine-

disrupting potential (Robin and Clanci, 2007; FDA, 2004). The exposure also disturbs the hormonal profile of species (Lafuente *et al.*, 2003; Hinson and Raven, 2006; Liu *et al.*, 2010; Cooke *et al.*, 2014). EDCs can influence behaviours and sex-specific development by affecting the HPG axis, which is involved in the release of GnRH which coordinates the reproductive maturation (Adewale *et al.*, 2009) and the hypothalamic-pituitary-adrenal axis, a stress mediator (Schug *et al.*, 2011). Another hormone cortisol is also secreted by the adrenal cortex that further activates the HPA axis (Schreier *et al.*, 2015). It has been observed that chemical and non-chemical stressors disrupt the HPA axis (Miller *et al.*, 2007; Cory-Slechta *et al.*, 2008).

Reports proposed that hypothalamic and pituitary functions are weakening by EDCs affect (Gore *et al.*, 2015; Kabir *et al.*, 2015). During steroidogenesis, EDCs interact with the HPA axis (Martinez-Arguelles and Papadopoulos, 2015). It has been observed that mercury (Hg), also alternates the function of the HPA axis (Tan *et al.*, 2009). Invertebrates stress indicator is cortisol that is released by HPA and HPI-axes (Barton, 2002; Heisler *et al.*, 2007). The higher concentration of cortisol increased the energetic costs (Leal *et al.*, 2011), effects process of metabolism (Bernier *et al.*, 2004), reproduction (Schreck, 2010; Fitzpatrick *et al.*, 2012), neurogenesis (Sørensen *et al.*, 2011) and immune function (Tort, 2011). Regulation of reproductive functions are also depending on the hypothalamus-pituitary–thyroid (HPT) and HPA axes i.e., the HPA axis modulates the performance of HPG axis (Dobson *et al.*, 2003). In the development period, reciprocal regulation or cross-talk of the HPT and HPG axes is another example of modulation of the axis (Hogan *et al.*, 2007).

Neurotoxicity

Environmental toxicant and EDCs induced neurotoxic effects as well as disrupt neurotransmitters synthesis, transportation and release of serotonin, dopamine, glutamate, and norepinephrine that play an important role in behaviour modulation learning, memory, cognition, and reproduction (Dickerson and Gore, 2007; Rasier *et al.*, 2007). At different stages of development, several neurons co-express steroid hormone receptors making them possible targets of EDCs (Mendola *et al.*, 2002; Dickerson and Gore, 2007). EDCs interrupting steroid-sensitive chemicals in the brain further affects learning, memory, cognition, metabolism, nonreproductive behaviours

as well as the system of reproductive neuroendocrine. Previous studies suggested that EDCs and non-EDCs exert synergistic and additive neurotoxic effects (Schug *et al.*, 2015). Ali et al. (1983) suggested that doses of food-based toxicant acrylamide elevated 5-HIAA in all regions of the brain. The studies also suggested that acrylamide increased the rates of serotonin (Aldous *et al.*, 1983; Ali *et al.*, 1983). Numerous findings specify that Pb & Cd disrupt the monoaminergic activity during CNS development (Bressler and Goldstein, 1991; Antonio *et al.*, 1996; Mejia *et al.*, 1997; Antonio *et al.*, 1998; Antonio *et al.*, 1999; Antonio *et al.*, 2002). Cadmium (Cd) an environmental toxicant is known to distress the levels of a neurotransmitter (Lafuente *et al.*, 2001). In previous reports, Cd exposure affected Dopamine (DA), Serotonin (5-HT), and noradrenaline (NA) (Lafuente *et al.*, 2001; Lafuente *et al.*, 2003). EDCs such as polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs) can modify the activity of serotonin (Clotfelter *et al.*, 2010; Rahman *et al.*, 2011).

It has been stated that polychlorinated biphenyl (PCB) affects the concentrations of serotonin binding to aryl hydrocarbon receptors (AhRs) and prevents tryptophan hydrolase (Boix and Cauli, 2012). Concomitantly, exposure of bisphenol A (BPA) elevates dopamine (DA) concentration in the medulla oblongata and dorsal hippocampus. However, the DOPAC/DA was decreased in different brain regions due to a reduction in monoamine oxidase B (Matsuda *et al.*, 2012). Higher stress levels disturbed the serotonergic activity in vertebrates (Winberg and Nilsson, 1993).

Various studies showed correlations of 5-HIAA/ 5-HT and plasma cortisol further suggested that 5-HT of the brain regulates HPI-axis in teleost homolog or HPA axis in mammals (Winberg *et al.*, 1997; Hoglund *et al.*, 2000) and HPA axis (Dinan, 1996; Heisler *et al.*, 2007). Serotonin also plays a significant role in the HPA axis regulation (Curtis and Patel, 2008). It has been observed that stress responses in nonprimates are also linked to the control mechanisms of the brain. However, serotonin, dopamine, and norepinephrine play a key role in coordination (Winberg and Nilsson, 1993; Chaouloff, 1993; Winberg *et al.*, 2001; Larson *et al.*, 2003; Lepage *et al.*, 2005; Perreault *et al.*, 2003). The serotonergic and dopaminergic activity of the brain involved in the regulatory mechanism of stress (Winberg and Nilsson, 1993; Bowman *et al.*, 2002; Perreault *et al.*, 2003; Lepage *et al.*, 2005) activated by a stressor (Schjolden *et al.*, 2006; Gesto *et al.*, 2009).

The teleost showed an elevated level of 5-hydroxyindoleacetic acid and 5-HIAA /5-HT in the brain (Alanärä *et al.*, 1998; Øverli *et al.*, 2001; Bowman *et al.*, 2002; Gesto *et al.*, 2013). Stressful events affect the synthesis of brain monoamines (Barton *et al.*, 2008) Øverli *et al.*, 1999). The 5-HT stimulates mammalians HPA-axis (Herman-Giddens *et al.*, 1997; Heisler *et al.*, 2007; Dinan, 1996;) and HPI-axis in fish (Øverli *et al.*, 1999; Hoglund *et al.*, 2000). Nevertheless, the role of central DA is still debatable in mammalians species. Dopamine has been considering to have no role in the HPA-axis mechanism (Sullivan and Dufresne, 2006; Brambilla *et al.*, 2000). Noradrenaline is also involved in triggering the mechanism and release of CRF, which further activates the mammalian HPA axis (Dunn *et al.*,2004).

In addition to steroids of gonads, numerous other aspects such as food, and neurotransmitters play a very important role in the regulation of the HPG axis, particularly the release of gonadotropin (Genazzani *et al.*, 2000; Zohar *et al.*, 2010). Monoamine, peptides, and amino acids also regulate the neuroendocrine mechanism of reproductive procedures (Gallo, 1980; Nock and Feder, 1981). Mammalians serotonin plays an extensive role in the regulation of reproductive mechanisms such as socio-sexual behaviours, gonadotropin release, secretion of GnRH, and maturation of gonads. Reproductive factors also modulate the system serotonin in the reverse mechanism. Serotonin may also be regulated by estrogen and progesterone (Pecins Thompson *et al.*, 1996). Mammalian serotonergic neurons are associated with beta receptors of estrogen (Gundlah *et al.*, 2001; Gundlah *et al.*, 2005). Central serotonin and signalling pathways of reproductive endocrine are closely associated (Prasad *et al.*, 2015). It is stated that the neuroendocrine system regulates reproduction with an assessment of the HPG-axis that is conserved in vertebrates (Prasad *et al.*, 2015). 5HT is associated with gonadotropins because the neurons of 5HT stimulate the release of gonadotropin via neuronal communication to the median eminence (Spinelli,2009). The axons of 5HT also end on the neurons of luteinizing releasing (LH) hormone in the preoptic region (Rubinow and Schmidt, 2006).

Given these studies, it is concluded that humans are exposed to furan in many ways either directly or indirectly. Hence, there is a need to perform a furan risk assessment on the reproductive toxicity of mammals using an appropriate approach. Moreover, limited data is available on the potential adverse effects of furan on the

reproductive systems (EFSA, 2009). However, due to complex structures, and regulatory functions, and physiology of the reproductive system, there is a high risk that exposure to toxicants induces many serious toxicological disorders at various sites of the reproductive tissues and organs (Creasy and Foster, 2002). Exposure to furan has been observed and exposure to this compound increases each day because of its formation in high amounts in home overcooked food and baby products. Currently, there is limited information and research data available about the actual mechanism of toxicological effects of furan on the reproductive systems. However, based on previous observation and studies on heat-induced food toxicants, we can hypothesize that furan will also affect the reproductive performance in terms of dysfunction of reproductive physiology and endocrinology including HPA-Axis, HPG-Axis, serotonergic and dopaminergic systems of rat.

AIMS AND OBJECTIVES

The long term goals of this research work were to examine the toxicological and endocrine disrupting potentials of furan in mammals. This research is unique to identify the effects of food-based toxicant furan, on the rats' reproductive system. To achieve the aim, the current study will be designed with the following objectives.

- To investigate the toxicological and endocrine-disrupting potential of furan in *rats*: an *in vitro* approach was used to target the process of androgenesis and oxidative stress in testis and epididymal sperms.
- To determine the toxic effects of furan experience *in vivo* on the male reproductive system through sub-chorionic toxicity study.
- To analyse the effects of furan on sperm production, maturation, and its functions to regulate or inhibit specific targets that are involved in infertility.
- To evaluate the consequence of furan on the neonatal period of life.
- To study the effects of furan exposure on HPG and HPA-axis: elucidated by determining plasma T, LH, FSH, cortisol concentrations and brain monoaminergic activity i.e., (5HIAA/5HT, DOPAC/DA, HVA /DA ratios, NE level).

- To find a link between furan and endocrine disruption: a bivariant correlation between T, LH, FSH concentrations, brain monoaminergic activity, NE level, and cortisol performed with known the effect of stress on reproduction.
- To find out the potential toxic effect of furan on reproductive performance of rats in F0 (parental) and F1 (offspring); a frequent oral dose-based furan administration in the extended one-generation study.

Chapter No. 1

In vitro assessment of different concentrations of food-based toxicant furan on testicular tissues and epididymal sperm cell of rats

ABSTRACT

Furan (C_4H_4O) is a volatile colorless toxic chemical, produced in various food processing and chemical industries. The current study was designed to find out the toxic effects of furan exposure in rat testicular tissues and epididymal sperm cells by using *in vitro* approach. For this purpose, rat's testicular tissue and epididymis sperm cells were properly incubated in Ham F12 media further supplemented with different concentrations 0, 1, 100, 500, and 1000 μg mL^{-1} of furan. The analysis revealed that furan generates high oxidative stress, and decreased antioxidant enzyme activity in the testicular tissue. Furan significantly lowered the concentration of testosterone (T) in (500 and 1000 μg mL^{-1}). Significant upsurge of ROS ($P<0.05$), lipid peroxidation ($P<0.01$), and decrease in CAT ($P = 0.01$), SOD ($P = 0.001$) and POD ($P = 0.04$) levels in 500 μg mL^{-1} and 1000 μg mL^{-1} furan treatment groups were observed. Similarly, 500 μg mL^{-1} and 1000 μg mL^{-1} treated groups induced a significant increase in the tail moment ($P=0.04$), % tail DNA ($P=0.1$), and the number of comets /100 cells ($P=0.01$) in sperm cells, when compared to the control group. Our results confirm that *in vitro* exposure to different concentrations of furan induces toxicity in testicular tissues and sperm cells through intracellular ROS production.

INTRODUCTION

Food processing at high temperatures has many benefits such as it improves the taste, color, texture, and reduces detrimental germs. However, many harmful compounds are produced during high-temperature food processing such as furan, acrylamide, etc (Durling *et al.*, 2007). In the case of humans, food is a common source of toxicant exposure (Pressman *et al.*, 2017). Furan is also one of the main chemical compounds used in several industrial sectors and is also produced in a variety of food items that experience heating processes such as coffee, sauces, soups, canned, jarred foods, infant formulas, and baby foods, etc (Bolger, 2009; EFSA, 2009; WHO, 2009; Bakhiya and Appel, 2010; Conti *et al.*, 2014; Cooke *et al.*, 2014; FDA, 2004b). Because of the toxic nature of furan and its excessive daily intake in humans through diet and air, it has become a matter of concern. The FDA has reported the presence of furan in food items in various surveys (FDA, 2004a, 2005). The EFSA reported that among adults, prepared coffee is the main source of furan in adults (EFSA, 2010). These reports revealed that the human diet contains an enriched furan and the occurrence of furan in food products has been confirmed. There is a need to investigate the possible toxicological effects of furan in the human body that might disturb the homeostatic balance of oxidant/antioxidant enzymes.

Various toxicants are dangerous to cell membranes since they cause an increase of lipid peroxidation (LPO), production of ROS, and cause DNA impairment in the cells (Farokhi *et al.*,2012; Cooke *et al.*,2014; Uçar and Pandir, 2017). Furan can easily pass through biological membranes due to its low polarity and acts as an endocrine disruptor EDC as it has been previously reported that exposure to different concentrations of furan resulted in the disturbance of the reproductive neuroendocrine system in rats (Cooke *et al.*,2014). Currently, very limited data on furan about reproductive toxicity is available (Cooke et al.,2014). Furan exposure to weaning rats results in decreased T and LH levels (Karacaoğlu and Selmanoğlu, 2010).

In vitro exposure to furan causes DNA damage in human sperm and lymphocyte cells through the generation of ROS (Pandir, 2015b). Comet assay methods have been used under *in vitro* conditions for the detection of DNA damage in the cell (Hickling *et al.*, 2010; Pandir, 2015b; Pandır, 2015). Hazardous toxicants can cause

variation in the cell's signalling pathways, defensive and protective systems of the antioxidant levels of the cells by affecting physiological conditions (Pandir, 2015b; Baş *et al.*,2016). *In vitro* investigation, exposure of furan to Chinese hamster ovary cells induces chromosomal aberrations and sister chromatid exchanges (NTP, 1993b). Thus, indicating a positive correlation between oxidative stress and furan exposure. Food toxicants induced oxidative stress, apoptosis, and damaged nuclear material by increasing the level of ROS (Mehri *et al.*, 2015; Pandir, 2015b).

Neuwirth *et al.* (2012) demonstrated that furan has DNA damaging and genotoxic nature (Neuwirth *et al.*, 2012). Similarly, exposure to furan caused apoptosis, cell proliferation, and impaired spermatogenesis ultimately affecting the reproductive functions (Karacaoğlu and Selmanoğlu, 2010). Based on previous observations as the stress-inducing capacity of furan, the current study was designed to evaluate the effects of furan on oxidative stress and DNA structure in rat epididymal sperm cells and testicular tissues *in vitro*.

MATERIALS AND METHODS

Animals and Chemicals

Sprague Dawley adults (80–90 days old) male rats were collected from Primate Facility, Department of Zoology, Quaid-i-Azam University, Islamabad Pakistan. The experimental animals were kept in steel cages (seven rats per cage) under the standard animal care facility of light (12/12) and at a temperature of (21 ± 2 ^{0} C). The rats had a free and easy approach to tap water in plastic bottles and laboratory feed. The experimentation procedures were undertaken according to the standard protocols approved by the Ethical Committee of the Department of Zoology, Quaid-i-Azam University Islamabad Pakistan. Furan (Catalog No.185922, 99% purity) was purchased from Sigma Aldrich, USA.

Experimental Design

Adult male rats (n=7) were utilized for testicular tissues and epididymal sperm cells. The experimental procedure of Wellejus *et al.* (2000) and Xu *et al.* (2001) was adopted with slight modifications. Different concentrations of furan, i.e., 0, 1, 100, 500, and 1000 μg mL^{-1} were used in this study. Dose selection was done according to studies on furan exposure to Chinese hamster ovary cells (NTP, 1993a), The different doses were carefully chosen rendering to OECD protocols. Furan concentrations were dissolved in dimethyl sulfoxide (DMSO) and finally diluted through cell culture media.

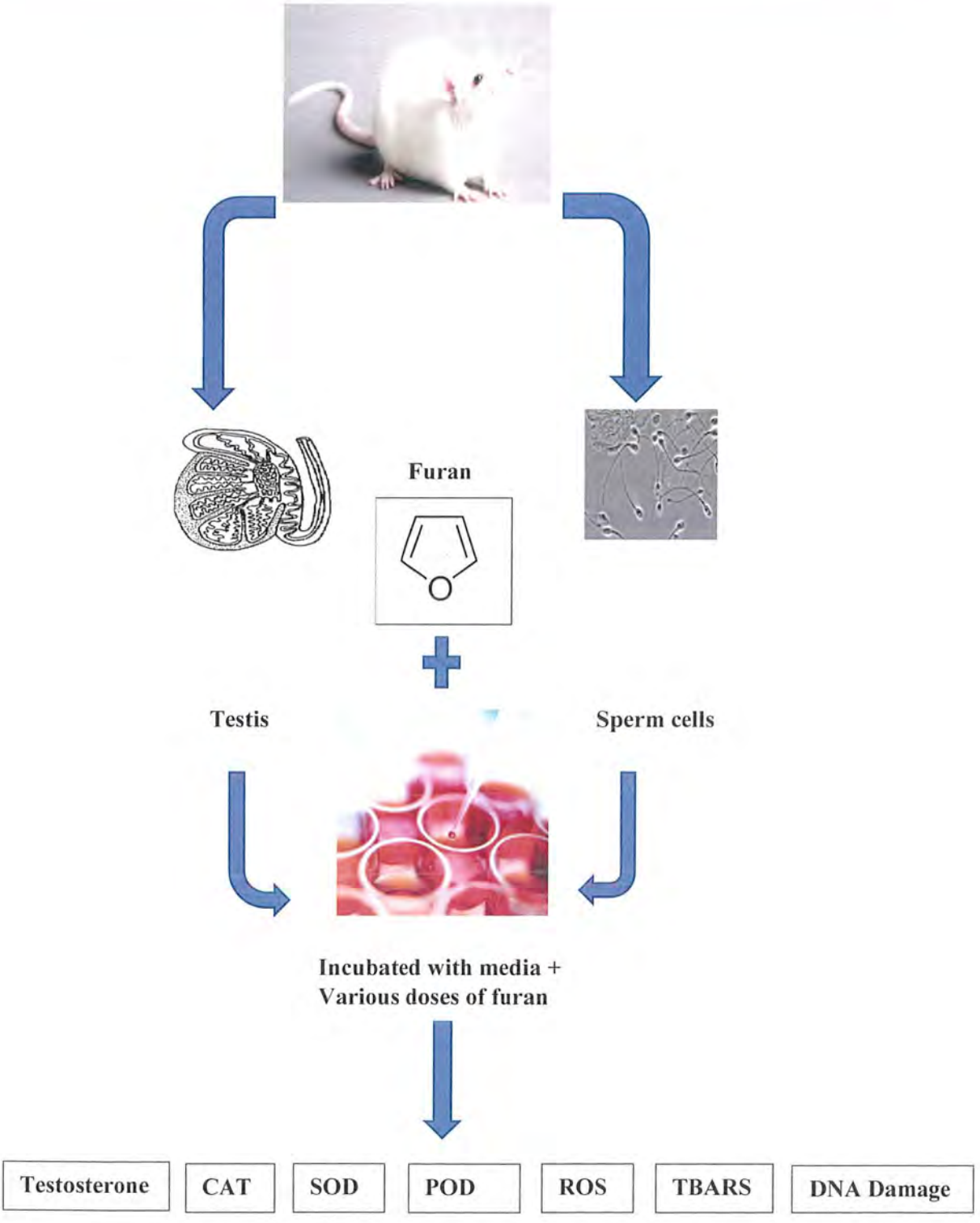


Fig 1. Schematic representation of *in vitro* furan exposure experiment.

Preparation of testis slices and incubation

The testicular tissues were cultured following previous protocols (Moundipa *et al.*, 2006; Freyberger *et al.*, 2010; Ullah *et al.*, 2016). Male rats were euthanized by decapitation. The testes individually were removed and cut into equal five parts and placed in the culture medium. The culture medium included 2 mL of Dulbecco's modified Eagle's medium/Ham F12 (DMEM/Ham F12 mixture medium) having 1.2 g L^{-1} sodium bicarbonate, 50 IU mL^{-1} penicillin, and 50 µg mL^{-1} streptomycin. Furan solution, at concentrations of 0, 1, 100, 500, and 1000 µg mL^{-1}, was added into the culture tubes, placed at 33 °C in a CO_2 incubator. After incubation for 2 hours, the tissues were removed from the CO_2 incubator and wash away with cold physiological saline. The tissues (90 mg) were homogenized in phosphate-buffered saline (PBS; 3ml) and centrifuged at 3,000 rpm for 0.5 hr. The supernatant was stored at -80 °C until it was utilized for analysis of hormones and t enzyme assays.

Preparation of sperm cells and incubation

For obtaining epididymis sperm, adult rats (n=7) were used. The experiment was performed, according to an earlier study of Xu *et al.* (2001) with some minor changes. After decapitation, the epididymis was quickly separated and washed in saline. The caudal part of the epididymis was dissected out and minced in 3 mL of a buffer (150 mmol/L NaCl, 10 mmol/L tris base, 10% glycerol, and 1 mmol/L EDTA, pH 7.4). After proper centrifugation at 4 ^{0}C for 10 min, the supernatant was removed and sperm was diluted with Ham's F-12 media. After that, 2 mL of these suspensions of sperm cells were put in culture tubes having either medium (2 mL) with <1 % DMSO only or the medium with a DMSO solution of furan at a concentration of 1, 100, 500,1000 µg mL^{-1} and all these culturing tubes were kept in a CO_2 (5 %) incubator at 35 ^{0}C up to 2 hrs. The samples were centrifuged (1000 rpm) for 10 min and the sperm pellet was used for the assays. Before assays analysis, 1 mL phosphate buffer was used for resuspension of sperm pellets. Half of the samples were placed at -80 ^{0}C until analysis of antioxidants and the remaining half sample was used for the comet.

The frozen samples of sperm were thawed and centrifuged up to 10 min at 4 ^{0} C. The supernatants were removed, and the pellet of sperm was diluted with PBS (50

mmol/L) containing EDTA (0.5 mmol/L) pH 7.0 with a concentration of 1×10^8 sperm/mL. The sperm homogenization was done in an ultrasonicator and the homogenate was used for the assessment of CAT (catalase), POD (peroxidase), superoxide dismutase (SOD), lipid peroxidation (LPO), and reactive oxygen species (ROS).

Antioxidant enzyme assay

Antioxidant enzymes along with CAT, POD, LPO, SOD, and ROS were determined from the supernatant of testicular tissues. The homogenate of sperm was used to estimate the antioxidant enzyme.

Catalase (CAT)

The method by Aebi (1984) was used to determine CAT activity. For this purpose, 2mL of phosphate buffer (pH of 7.0) was used for dilution of 50mL of homogenate. After mixing, the 240-nm wavelength was used to read the absorbance within 15 and 30 seconds.

Superoxide dismutase (SOD)

SOD activity was measured according to the standard protocol as described by Kakkar *et al.* (1984). The amount of chromogen formed at 560-nm was used to determine SOD activity. The results were reported in mU /10^8 sperm.

Peroxidase (POD)

In this assay, guaiacol (0.1mL), H_2O_2 (0.3mL), and phosphate buffer (2.5 mL) were mixed with 0.1mL of sperm homogenate. The wavelength of 470-nm was used to read absorbance. One unit of POD means a change in the absorbance 0.01 units/min (Carlberg and Mannervik, 1975).

Thiobarbituric acid reactive substances (TBARS)

TBARS is an indicator of LPO, that was measured as previously described by Ohkawa *et al.* (1979). Testes and sperms homogenized samples were mixed with Tris–HCl buffer (10 mL; 150 mmol/L, pH 7.1), 10 mL ferrous sulfate (1.0 mmol/L), 1 mL ascorbic acid (1.5 mmol/L), 60 mL water and incubated for 15 min at 37 °C. After the incubation procedure, the reaction was stopped by the addition of trichloroacetic acid (10% w/v) and then incubated at 100 ^{0} C for 15 min after adding thiobarbituric acid (0.2 mL; 0.37 % w/v). Finally, the samples were centrifuged (1000 rpm) for 10 min. Measured the optical density at 532 nm is the amount of TBARS in each sample and expressed as nmol malonaldehyde/min/10^8 spermatozoa at 37 ^{0} C using a coefficient of 156 mmol/L/cm.

Reactive oxygen species (ROS)

Hayashi *et al.* (2007) method was used to measure ROS in the homogenate. To a 96 well microplate, 140 µl of sodium acetate buffer (0.1 Mol/L; PH 4.8) was added to each well of the plate. Standards of 5 mL homogenate of sperm or H_2O_2 (30 % w/w) were prepared by consecutive dilutions (0.23, 0.46, 0.92, 1.87, 3.75 and 7.50 mg H_2O_2). After that, these standards were put in wells of the plate and further incubated for 5 min at 37 ^{0} C. Then 100mL of the mixed solution of N, N-diethyl-para-phenylenediamine, and ferrous sulfate (1: 25) was added and incubated 60 seconds. The absorbance was taken at 505 nm wavelength for 180 sec with 15-sec intervals using a microplate reader. A curve was plotted, and the ROS concentrations were reported in unit/10^8 sperm. In the sample, the unit of ROS was equal to levels of H_2O_2 (1-unit = 1.0 mg H_2O_2/L).

Assessment of DNA damage

The DNA damage of sperm was measured by using the neutral comet assay with some slight modifications as previously reported by Boe-Hansen *et al.*, 2005.

Hormonal analysis

Intratesticular testosterone (T) concentrations were quantified using ELISA kits (Cat # BC-1115, Bio Check Inc., USA) according to standard protocol instructions and information delivered by the manufacturer inside the kit.

Statistical analysis

The results were expressed as (mean ± SEM). All statistical data were analyzed by using eassyanova (Arnhold, 2013) and lme4 (Bates *et al.*, 2014) package of R 3.2.5 (R Development Core Team, 2016). The effects of different treatments were judges by ANOVA ea1 command of R with a completely randomized design as followed by post hoc *Tukey's HSD*. The values of $P<0.05$ were considered here as statistically significant.

RESULTS

Effect of *in vitro* furan exposure on testicular tissues after incubation with different furan concentrations

Activities of SOD, CAT, and POD in rat testicular tissue were decreased compared to control as shown in Table 1. CAT activity significantly increased as the dose of furan increase in the treatment groups, (Table 1, Fig 2). SOD level did not show statistical differences in any treatment group compared with control (Table 1, Fig 2). Significantly ($P<0.001$) decreased levels of POD were also observed in the 500-$\mu g\ mL^{-1}$ and 1000 $\mu g\ mL^{-1}$ dose groups when compared with control. Substantial differences were also observed in ROS and LPO levels ($P=0.08$) compared to non -treated group (Table 1, Fig 2).

Change in intratesticular testosterone concentration (ng/g tissue) was measured after 2 h of incubation with various doses of furan. Testosterone concentration was significantly ($P=0.03$) decreased in the 1000 $\mu g\ mL^{-1}$ dose groups. However, a non-significant decrease in T concentration was also noticed in other treatment groups (Table 1, Fig 3).

Table 1. Effect of *in vitro* furan exposure on testicular antioxidants and intratesticular T concentration of rats.

		Furan (μg mL^{-1})				Statistics
Parameters	**Control**	**I**	**100**	**500**	**1000**	**P and F values**
CAT (U mg^{-1} protein)	22.72±2.58[a]	18.33±2.63[ab]	15.07±0.71[ab]	12.97 ±0.57[b]	11.86 ± 0.29[b]	P<0.001, F=6.32
SOD (U mg^{-1} protein)	18.82±4.40[a]	16.17±2.12[a]	16.31±0.97[a]	13.71±1.13[a]	10.32±1.6[a]	P=0.1, F=1.77
POD (nmol)	4.82±1.53[a]	4.08±0.44[c]	3.74±0.79[c]	1.95±2.7[b]	1.02±1.5[c]	P<0.001, F=128.36
LPO (nM TBAR min^{-1} mg^{-1})	26.57±2.58[a]	44.85 ±7.79[a]	45.14±8.62[a]	53.42 ±8.17[a]	51 ±6.24[a]	P=0.08, F=2.22
ROS (U g^{-1} tissue)	35.85 ±3.28[a]	38.57 ± 1.21[a]	40.14±2.62[a]	56 ± 1.67[a]	59.57±2.27[a]	P=0.1, F=1.29
T (ngg^{-1} tissue)	68.14± 2.43[a]	53.28 ± 1.97[ab]	47 ± 2.90[ab]	49.71 ± 2.90[ab]	25.71 ± 1.34[b]	P=0.06, F=2.48

P and F values in the rows were obtained from ANOVA with completely randomized simple designs, followed by Tukey's post hoc analysis, which shows a pairwise comparison of control and furan-treated groups. Mean values with different superscripts show significant differences (P<0.05) in the columns when comparing control with other treatment groups.

[a]Values are presented as Mean ± (SEM)

[b]Significance at P<0.05 vs control

[c]Significance at P<0.001 vs control

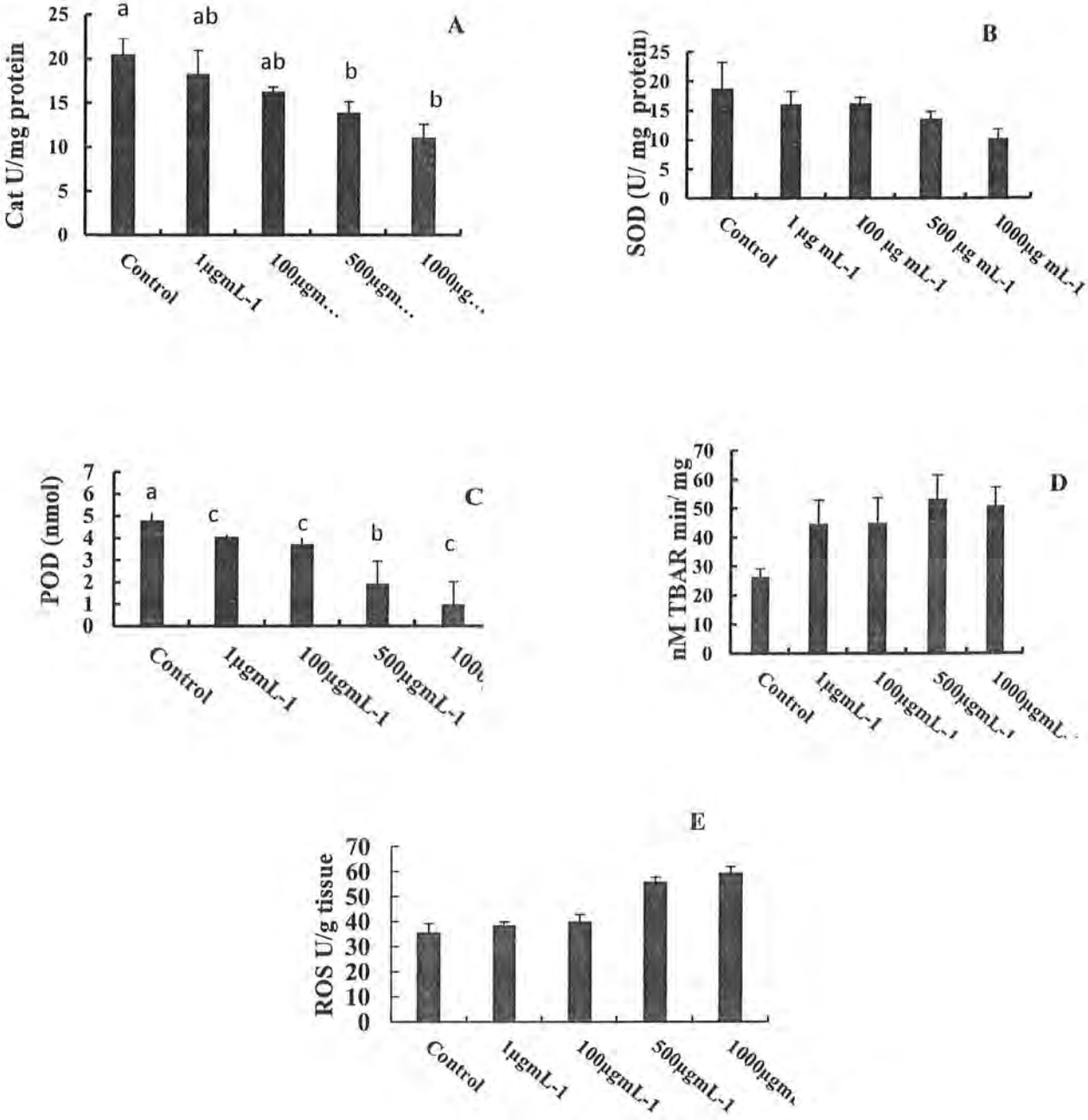


Fig 2. Effect of *in vitro* furan on (A) CAT, (B) POD, (C) SOD, (D) TBARS, (E) ROS enzymatic levels of rat testicular cells. Values represent mean ± SEM. Letters above bars indicate values that are significantly different ($P<0.05$) between different doses within the testicular tissue.

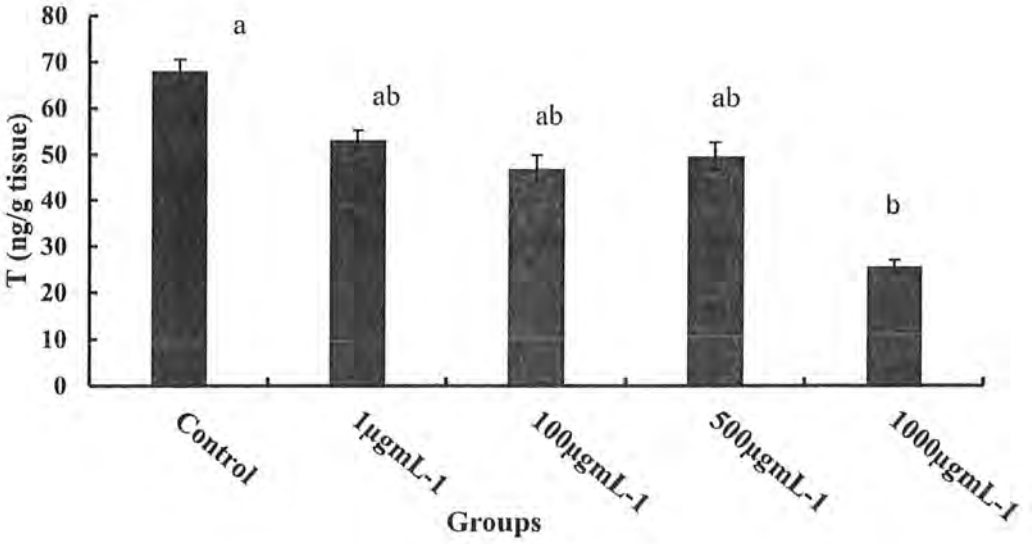


Fig 3. Effect of *in vitro* furan exposure on intratesticular testosterone (T) level in rats. Values represent as mean ± SEM. The letters above bars depict values that are significantly different ($P<0.05$) between different doses.

***In vitro* sperm incubation at different concentration of furan**

The antioxidant enzyme activities were monitored to evaluate the antioxidant level in the spermatozoa. There was a significance decrease in CAT (P = 0.01), SOD (P = 0.001) and POD (P = 0.04) activities in 500 μg $mL^{-1}$and 1000μg mL^{-1}treated groups compared with control group (Table 2, Fig 4). On contrary, we observed a significant (P=0.01) upsurge in TBARS activity in the 1000 μg mL^{-1} treated group. The 1000μg mL^{-1}Furan treated group had shown 1.39±0.37 nM Malenoaldehyde/min /10^8 spermatozoa as compared to control with values of 0.44±0.07 nM Malenoaldehyde/min /10^8 spermatozoa. However, the lower doses treated groups showed no significant variations similar to the non-treated group (Table 2, Fig 4). Furthermore, an increase in spermatozoa intracellular ROS (P=0.04) level was observed. Higher doses of 500μg $mL^{-1}$and 1000μg mL^{-1} (1.25±0.35 U/10^8, 1.44±0.34 U/10^8 cell) showed countable elevation of ROS production. While intracellular ROS levels remained the same in low dose treated groups (Table 2, Fig 4).

Table 2: Effect of *in vitro* furan exposure on antioxidants level of epididymis sperm cells.

	Furan (µg mL^{-1})					
Parameters	**Control**	**I**	**I00**	**500**	**I000**	**Statistics**
CAT (U/10^8 cell))	25.57±3.73^a	25.39±2.72^a	22.23±1.81^{ab}	23.86±1.23^{ab}	19.63±1.25^b	P<0.001 F=86.90
SOD (mUnit/10^8 cell)	10.28±0.59^a	9.54 ±0.98^{bc}	7.14±0.82^{bc}	6.29±0.55^c	5.54±0.71^c	P<0.001 F=62.51
POD (nmole/10^8 cell)	11.98±0.64^a	9.55±0.77^{ab}	7.13±1.33^{ab}	8.32±0.87^b	6.29±0.86^b	P<0.001 F=39.80
LPO (nmol/10^8 spermatozoa)	0.44±0.07^b	0.49±0.11^b	0.57±0.41^{ab}	0.74±0.24^{ab}	1.53±0.25^a	P<0.001 F=62.51
ROS (U/10^8 cell)	0.20±0.14^c	0.39±0.19^{bc}	1.57±0.31^{ac}	2.52±0.08^{ab}	4.29±0.42^a	P<0.001 F=35.64

P and F value in the rows from ANOVA followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P<0.05) in the rows. Values are presented as Mean ± (SEM).

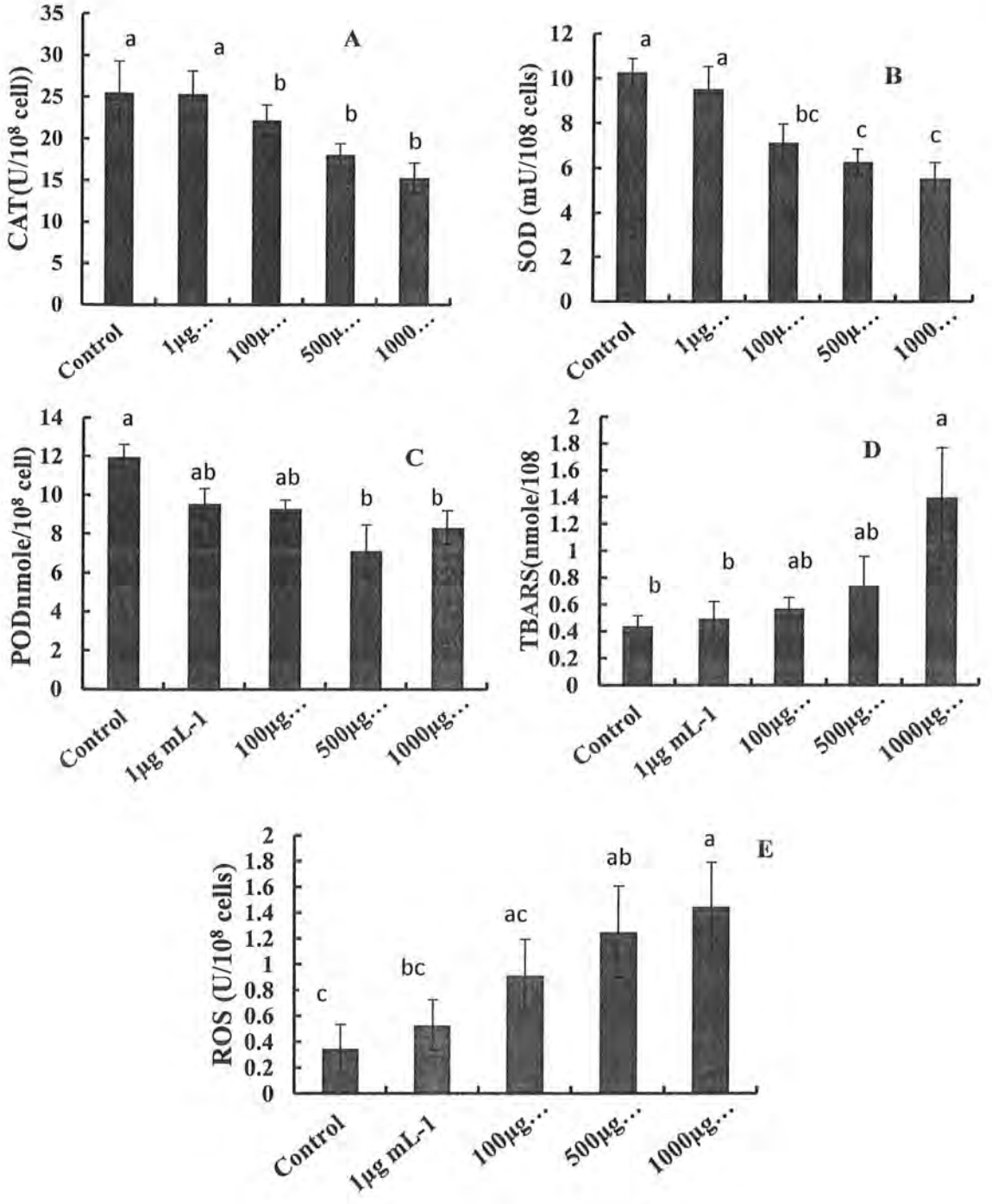


Fig 4. Effect of *in vitro* furan exposure on (A) CAT, (B) POD, (C) SOD, (D) TBARS, (E) ROS enzymatic level in rat sperm cells. Values represent mean ± SEM. The letters above bars depict values that are significantly different ($P<0.05$) between different doses within the same sperm cells.

DNA damage

Results of DNA damage after furan exposure are presented in Fig 5. Sperm treatment with a high concentration of furan showed more DNA fragmentation compared to control. Table 3 showed comet assay parameters in various treatment groups. High doses groups 500 μg mL^{-1} and 1000 μg mL^{-1} showed countable increase in the tail moment (P=0.04), tail DNA % (P = 0.1), and the number of comet /100 cells (P = 0.01) in sperm cells in Table 3, Fig 5.

Table 3. Effect of *in vitro* furan exposure on epididymal sperm cells after 2 hours incubation showing the value of Tail DNA (%), Tail moment, and No of comet /100 cells in control and treated groups.

	Furan (μg mL^{-1})					Statistics
Parameters	**Control**	**1**	**100**	**500**	**1000**	**P and F value**
Mean ±SEM Tail moment (μm)	5.03±0.79[a]	5.04±2.45[ab]	7.71±0.67[ab]	8.35±1.06[ab]	7.48±1.57[b]	P=0.04 F=2.31
Mean ±SEM Tail DNA (%)	12.11±0.69[a]	12.57±1.56[a b]	14.11±1.11[a b]	16.76±1.16[a b]	18.51±3.37[b]	P=0.1 F=1.83
Mean ±SEM No of comet /100cells	14.66±1.18[a]	16.25±0.56[a b]	18.66±0.91[a b]	19.33±1.94[a b]	21.08±1.62[c]	P=0.01 F=3.60

P and F value in the rows from ANOVA followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscripts are significantly different (P<0.05) in the rows compared to control with other treated groups.

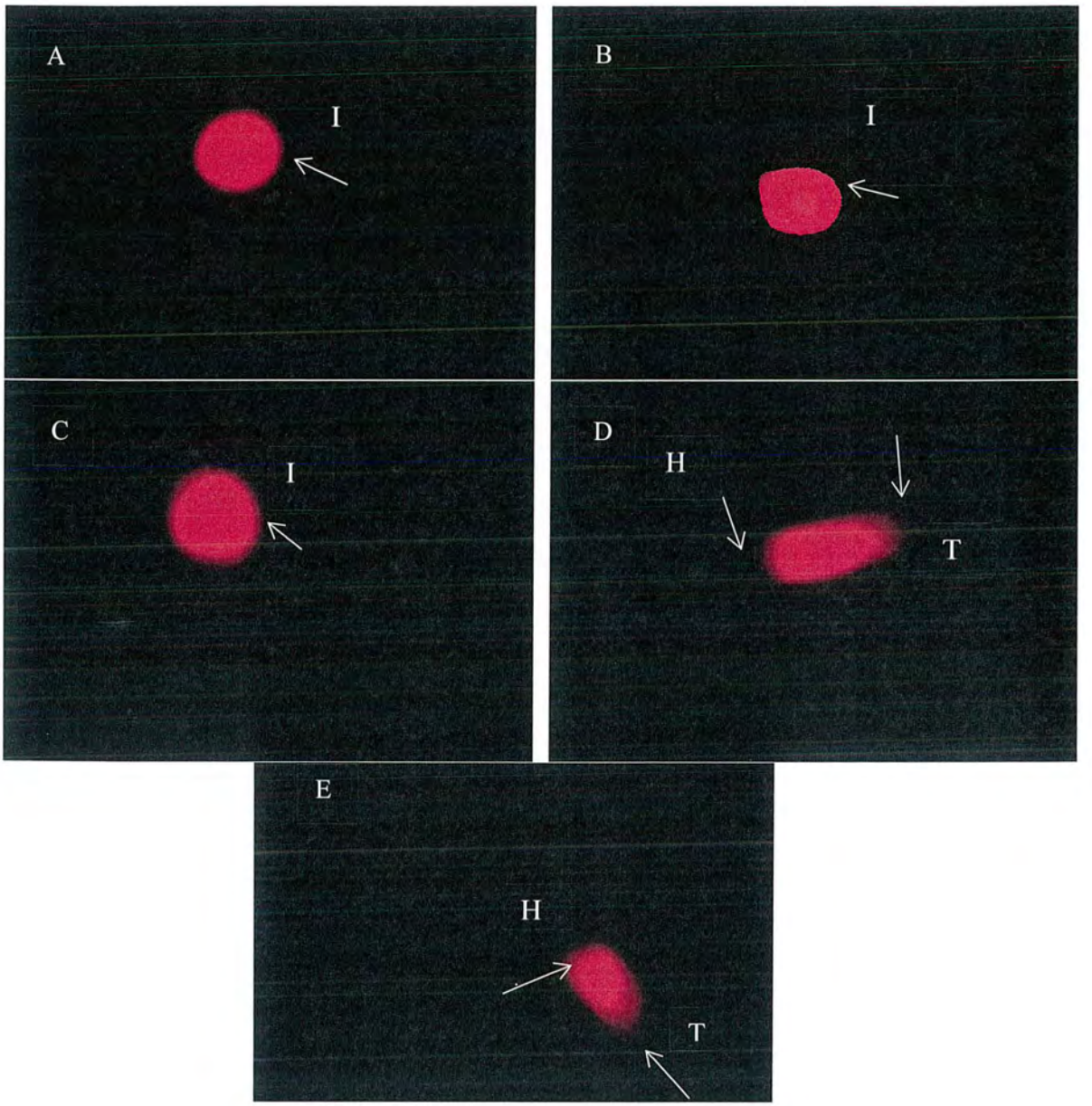


Fig 5: Effect of *in vitro* furan exposure on the total length of chromatin dispersion in the sperm cells treated with (A) control group, (B) 1 μg mL^{-1} group (C) 10 μg mL^{-1} group (D) 100 μg mL^{-1} group, and (E) 1000 μg mL^{-1} group at 40 X. Intact (I), Head (H), Tail (T).

DISCUSSION

Limitations have been enforced on distinct environmental chemicals, due to their possible health consequences and endocrine-disrupting capabilities. Furan is a food-based environmental toxicant, that is founded from numerous pioneer molecules easily present in food during several steps of treating, which might include oxidation or Maillard reaction (Limacher *et al.*, 2007; Limacher et al., 2008; Bolger, 2009). It has been earlier described that furan is a toxic agent that produces numerous damaging impacts on the biological systems of living organisms (Gill *et al.*, 2010; Jackson *et al.*, 2014; Webster *et al.*, 2014; Pandir, 2015a; Uçar and Pandir, 2017). Furan gained more consideration of the scientist because of its endocrine disrupting potentials and carcinogenic nature (Karacaoğlu and Selmanoğlu, 2010; Pandir, 2015a). Previous literature on furan reported that its exposure causes reproductive impairment by disturbing spermatogenesis, thereby leading to apoptosis in germ cell lining and Leydig cells. Reproductive organs are important parts of our body and any disease or issues related to the testis or epididymis could be considered a serious health issue. Among carcinogen food contaminants furan also disturbs the reproductive functions of male rats (Cooke *et al.*, 2014; Rehman *et al.*, 2019). Therefore, *in vitro* study was intended to find out the toxic effects of furan on spermatozoa cell's antioxidant enzyme activity, ROS, sperm production and DNA impairment. In the *in vitro* experiment direct impact of furan exposure on ROS accumulation, oxidative stress, lipid peroxidation, and DNA damage of testicular tissues and spermatozoa cells and were observed.

The results of *in vitro* study showed decreased levels of antioxidants in testicular tissue. While (ROS) and (LPO) levels were elevated in testicular tissues by furan treatment compared to the non-treated group. These results are similar to previous findings as furan and acrylamide exposure resulted in an elevated level of LPO and (ROS) (Mehri et al., 2012; Zhao et al., 2013; Pandir, 2015a). All these variations might be due to high ROS production. ROS are Oxygen-containing molecules having free electrons and are highly reactive. Certain nonradical molecules also act as ROS. They produce free oxygen ions during the normal metabolic process. Oxidative phosphorylation occurs in mitochondria,

which results in the production of ROS (Devasagayam *et al.*, 2004). Elevated ROS construction imparts damaging effects on protein, lipids, and DNA (Radak *et al.*, 1999). The self-defence antioxidant enzyme mechanism is triggered in response to ROS production, which reduces the levels of ROS production (Kaul and Forman, 2000). The decrease in antioxidant enzyme overcomes the cell's sensitivity to harbour oxidative stress, hence compromising its ability to detoxify the effects of ROS (Kaul and Forman, 2000; Pérez *et al.*, 2009). Previously, furan was found to be a cytotoxic, genotoxic, and an apoptotic inducer (Heppner and Schlatter, 2007; Karacaoğlu and Selmanoğlu, 2010).

In the present study, T concentration decreased in groups exposed to high doses of furan. However, a decreased level of T is an indicator of chemical toxicity (Yoshida *et al.*, 2002). As previously described, reduced T concentration is due to oxidative stress, which is connected to the main action of antioxidant enzymes within Leydig cells and enhances the sensitivity of spermatogenesis toward oxidative stress (Cao *et al.*, 2004; Rezvanfar *et al.*, 2013). From the present study, it is concluded that Furan exerts its effects by induction of oxidative stress that also alters T secretion in testis, resulting in decreased sperm production and spermatogenic arrest.

Previously reported that ROS production induces cytotoxicity and cell damage (Pandir, 2015b).In rats and rabbits, ROS generation is responsible for the suppression of sperm movement and sperm loss by spermatozoa leaking electrons from the mitochondrial electron chain, resulting in the production of ROS (Vernet *et al.*, 2001; Aitken and Baker, 2004).In the sperm plasma membrane, a high concentration of fatty acids is present (Aitken *et al.*, 1995) and higher ROS production causes damage to membrane lipids, disturbing sperm production and functions (Aten *et al.*, 1992; Ichikawa *et al.*, 1999; Zalata *et al.*, 2004). (Aten *et al.*, 1992; Ichikawa *et al.*, 1999; Zalata *et al.*, 2004). High ROS production reveals harmful impacts on protein, lipids, and DNA (Radak *et al.*, 1999). Our current findings from *in vitro* approached have shown spermatozoa incubation with furan decreased the antioxidant enzyme activity of cells. While ROS and LPO levels were increased in spermatozoa cells. Previously it has been reported that acrylamide induces ROS production and lipid peroxidation in PC12 cells in animal models (Yousef and El-

Demerdash, 2006; Rodríguez-Ramiro *et al.*, 2011b; Cao *et al.*, 2008; Rodríguez-Ramiro *et al.*, 2011a; LoPachin and Gavin, 2015).In the current study, sperm DNA damage was seen in a group exposed to a high dose of furan which might be due to the elevated level of ROS. These findings are similar with previous reported work in which dose-dependent increase in comet tail, tail length as well as tail moment were observed in human lymphocytes and spermatozoa cells *in vitro* (Pandir, 2015b).

As an important sperm quality indicator, sperm DNA damage assessment that can affect fertility has gained special attention (IRVINE *et al.*, 2000). From our current results, it seems that furan significantly increases the number of DNA fragmented cells in the treated versus a control group that acts as a marker of apoptosis. Our results are in line with previous studies of *in vivo* cell systems where furan has been reported to act as a genotoxic and apoptosis inducer (Neuwirth *et al.*, 2012; Uçar and Pandir, 2017). Current findings of the increased DNA damage in the spermatozoa and decreased DSP suggest the detrimental effects of food-based toxicant furan on the reproductive physiology of male rats.

CONCLUSION

The current study elaborates the oxidative stress-inducing capability as well as the genotoxic potential of furan in testis and sperm *in vitro* experimentation. All these effects of furan might be the ROS and LPO production. The current investigation showed that food-based toxicants, furan induce oxidative stress, DNA damage that leads to diminished sperm quality. It is suggested here that furan might harm germ cells and suppress fertility by producing oxidative stress and damaging sperm DNA. Moreover, further studies are recommended to properly investigate the basic phenomenon of furan toxicity in the sperm as well as in steroidogenic cells.

Chapter No. 2

Effect of sub-chronic furan exposure on the male reproductive system of rats - A histological and biochemical based study.

ABSTRACT

Furan is a colourless heterocyclic organic compound produced in various chemical industries and also formed in various food items during heating processes, such as coffee, sauces, soups, canned, jarred foods, infant formulas, and baby foods. Based on results from our previous *in vitro* study an *in vivo* subchronic exposure study was performed. In the present study, an *in vivo* experiment was intended to measure the effects of furan exposure on oxidative stress, hormonal concentration, and brain monoamines level in rats. The male rats were orally administered to various concentrations of furan 5, 10, 20 and 40 mg kg^{-1}. A significant ($P<0.05$) increase was observed in the generation of reactive oxygen species (ROS) and lipid profile (cholesterol, triglycerides and LDL) in higher furan dose treatment groups, while the total protein content and antioxidant enzyme activity were considerably decreased. Reduction in plasma and intratesticular T concentrations were noticed in high-dose treatment groups. Plasma LH and FSH concentrations were decreased in 20 and 40 mg kg^{-1} treated groups. Contrarily, a significant increase in plasma cortisol concentration, brain monoamines (5HIAA/5HT, DOPAC/DA and HVA /DA) ratios and NE levels were evident in the high dose groups, 20 mg kg^{-1} and 40 mg kg^{-1}. The sperm variables such as sperm viability, count, and motility showed a decrease ($P<0.05$) in a dose-dependent manner. The histopathological observations revealed significant alterations in testis and epididymis tissues. Our present findings confirm that furan can induce adverse effects on the reproductive system of male rats.

INTRODUCTION

Furan is a colourless heterocyclic organic compound produced in various chemical industries and also formed in a variety of food items during heating processes, such as coffee, sauces, soups, canned, jarred foods, infant formulas, and baby foods (Bolger, 2009; EFSA, 2009; WHO, 2009; Bakhiya and Appel, 2010; Conti *et al.,* 2014; Cooke *et al.,* 2014; FDA, 2004). Furan is also present in the environment as the main component of cigarette smoke, wood smoke, and exhaust gases from engines. Furan needs to be removed from consumer use due to its harmful effects on human life and health. Previous observations reported that furan caused various types of cancer and have a toxic effect on the biological system of a living organism (IARC, 1995; Hamadeh *et al.,* 2004; Pandir, 2015; Uçar and Pandir, 2017). This compound induces liver and hepatic necrosis and DNA damage, as well as associated with an elevated level of alanine aminotransferase (ALT) in the rat's serum (Gill *et al.,* 2010; McDaniel *et al.,* 2012). The metabolite of furan promotes the induction of cellular proliferation and disconnection of oxidative phosphorylation of mitochondrion (Kedderis, 1999). Bas and Pandir (2016) reported that furan exposure increases malondialdehyde (MDA) in rats, while superoxide dismutase (SOD), glutathione-S-transferase (GST), and catalase (CAT) were decreases in furan treated groups (Bas and Pandir, 2016). Furan caused significant changes in histological structure, DNA structure of ovarian cells, malondialdehyde levels, and antioxidant enzyme activities (Uçar and Pandir, 2017). In a human study, furan exposure generated reactive oxygen species and affected lymphocytes and sperm (Pandir, 2015). Although, very limited information is available in the literature about the adverse effects of furan on the male reproductive mechanism and brain monoamines levels.

The hypothalamus-pituitary gonadal axis (HPG) of vertebrates is controlled by various internal or external factors (Prasad *et al.,* 2015). The factors such as stress, steroids, nutrition, monoamine, amino acids and peptides play important role in the regulation of the HPG axis that further control the neuroendocrine mechanism of reproduction (Genazzani *et al.,* 2000; Zohar *et al.,* 2010). The brain of vertebrates plays a vital role in the control of reproduction (Kah *et al.,* 1993). In a brain (CNS system) several neurotransmitters, like 5- hydroxytryptamine (5-HT), dopamine (DA) and noradrenaline

(NA) are the important monoamines, and their functional role in stressful conditions (Tsigos and Chrousos, 2002) and sexual functions (Giuliano and Rampin, 2000; Heaton, 2000). Dysfunction of these monoamines has been related to a wide-ranging of central and peripheral disorders and dysregulation of HPG and HPA axis (Sheikh *et al.*, 2007; Prasad *et al.*, 2015).

Many environmental and food toxicants crossed the blood-brain barrier and caused neurotoxicity (Leret *et al.*, 2003; Yadav *et al.*, 2010; Matsuda *et al.*, 2012). Previously declared by the FDA that food-based contaminants furan and acrylamide, having endocrine-disrupting potential (Robin and Clanci, 2007; FDA, 2004). The exposure also disturbs the hormonal profile of the species (Lafuente *et al.*, 2003; Hinson and Raven, 2006; Liu *et al.*, 2010). It has been observed that EDCs influence behaviour and sex-specific development by effecting HPG-axis, that manages reproductive maturation through GnRH (Adewale *et al.*, 2009). The HPA-axis also mediates the stress response (Schug *et al.*, 2011). Another hormone cortisol is secreted from the adrenal cortex following the activation of the HPA- axis (Schreier *et al.*, 2015). HPA axis is potentially disturbing by non-chemical (social stressors) and chemical factors (Miller *et al.*, 2007; Cory-Slechta *et al.*, 2008). The neuroendocrine also regulates the HPG-axis that conserved evolutionarily in vertebrates (Prasad *et al.*, 2015). Neurotransmitters also control the reproductive mechanism (Gallo, 1980; Nock and Feder, 1981). Mammalians serotonin is also involved in an extensive control of social-sexual behaviours, secretion of GnRH, gonadotropin release and maturation of gonads. Ovarian steroids estrogen and progesterone also regulates serotonin secretion in the mammalian brain (Pecins-Thompson *et al.*, 1996). Reproductive endocrine signaling pathways and brain Serotonin are associated closely with each other (Prasad *et al.*, 2015) .

Given these studies, it is concluded that humans are exposed in many ways, either directly or indirectly. Hence, there is a need to perform a furan risk assessment on the reproductive toxicity of mammals using an appropriate approach. Moreover, limited data and information are available about the potential effects of furan and other carcinogenic compounds reproductive functions of animals (EFSA, 2004). However, due to complex

physiological organs and regulatory functions of the reproductive system of male, there is a high risk that exposure to toxicants induces many serious toxicological disorders at various sites of the reproductive tissues and organs (Creasy & Foster, 2002). Therefore, the current investigation was commenced to elucidate the possible effects of furan exposure on the mammalian reproductive system and brain monoamine levels.

MATERIALS AND METHODS

Chemicals and Animals

The animals were maintained according to the standard protocol as described above in chapter 1.

Experimental design

The current experiments were properly designed to study the effects of furan on the reproductive system of male rats. *In vivo* experiment, a sub-chronic exposure study was performed for 28 days to assess the effects of furan on male rats' reproductive performance. Experimental animals were also observed to notice any morphological and physiological variations during the experimental period.

In vivo sub-chronic exposure experiment

Adult male rats (n=35) aged 80-90 days old were selected. The animals were divided into five groups (n=7 rat/group). A standard solution of furan was ready in corn oil. Furan was orally administered through different concentrations 0, 5, 10, 20 & 40 mg kg^{-1} for 28 days. Dose time was chosen according to the standard protocol 407 of the OECD (2008). Different doses of furan were selected to assess the dose-dependent sub-chronic exposure-response. Furan doses selected for this experiment was based on the doses used in previous studies on furan and acrylamide with some slight modifications in concentration (Rawi *et al.,* 2012; El-Akabawy and El-Sherif, 2016; Kara *et al.,* 2016; Dortaj *et al.,* 2017) and OECD guideline # 408 (doses can be used according to the relevant substance). On day 29, rats were decapitated; blood samples collected from the trunk region with pre heparinized syringes, and reproductive organs (testis, epididymis, seminal vesicle and prostate) were separated and weighed using, weighing balance (AND GF-300, JAPAN) and stored for further analysis.

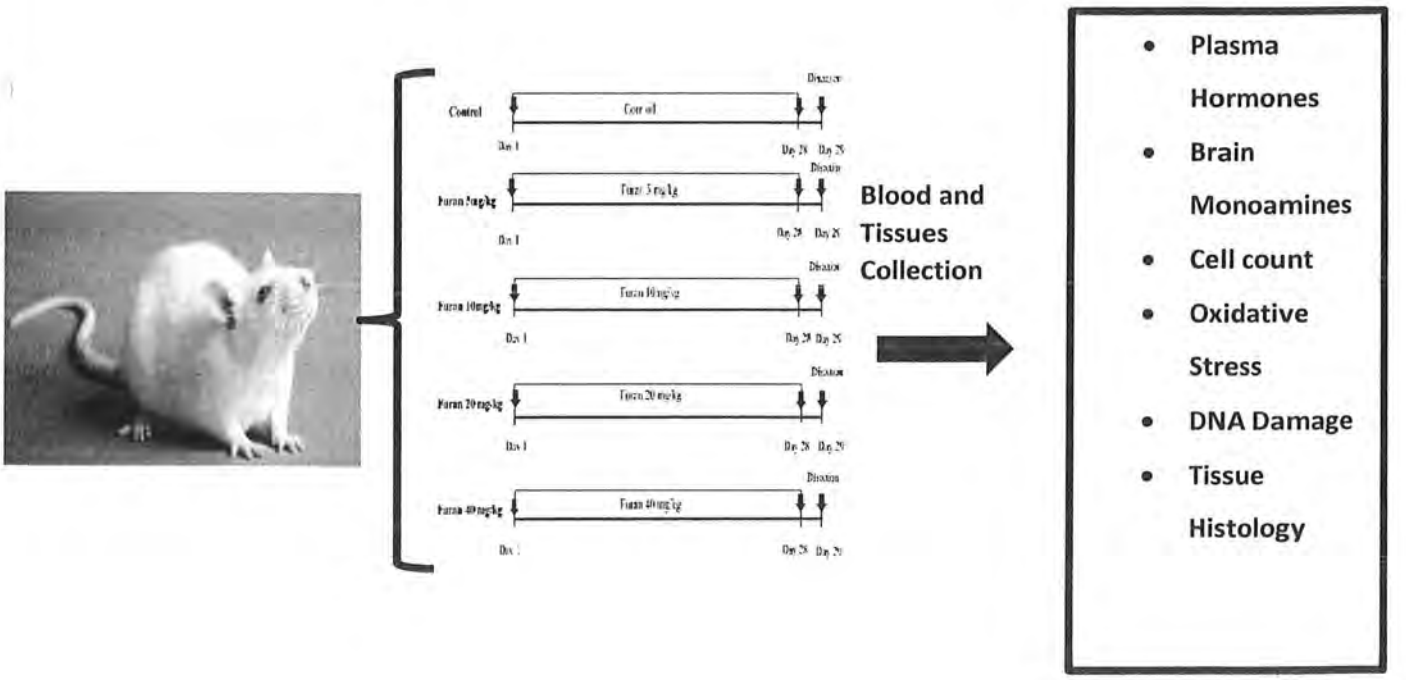


Fig 6. Schematic representation of *in vivo* sub-chronic furan exposure in rats.

Blood and tissue collection

The collected blood samples were centrifuged (3000 rpm) for ten minutes. After centrifugation, the blood plasma was collected and frozen at -20 °C for further hormonal investigation. After dissection, the left testis and epididymis were stored in liquid nitrogen for further investigation while the right testis and epididymal tissues were fixed in formalin (10%) for histological observation.

Antioxidant enzyme assay:

The tissues were collected from *in vivo* experiment and processed for antioxidant enzyme analysis. The collected tissues from experiments were homogenized in PBS and centrifuged for at least 30 min at 3,000 rpm. The supernatant was collected for protein estimation, antioxidant enzyme assay and hormonal analysis.

Antioxidant status of the testicular tissue was determined using the supernatant collected from *in vivo* experiments. The activity of the plasma antioxidant enzymes catalase (CAT) (Aebi, 1984), superoxide dismutase (SOD) (Kakkar *et al.*, 1984), and peroxidase (POD) (Carlsberg, 1975); lipid peroxidation (TBARS) ; (Iqbal *et al.*, 1996) and reactive oxygen species (Hayashi *et al.*, 2007) were assessed.

Total protein

The protein contents in the tissues were quantified through protein kit of AMEDA Labordiagnostik GmbH, Krenngasse, Graz, Austria (Cat # BR5202-S). The total protein was assessed by plotting the absorbance of kit standards versus the sample absorbance. The total protein contents were stated in mg g^{-1} of tissue.

Analysis of serum biochemistry

Blood plasma was analyzed for total cholesterol (Cat # REF2230650), triglycerides (Cat # REF4730650), HDL (Cat # BR3251), LDL (Cat # BR3302) and AMP kits (AMEDA Labor Diagnostic GmbH, Austria) by using a chemistry analyzer according to manufacturer's instruction manual inside the kits.

Sperm parameters

Sperm motility

For sperm motility assessment, the epididymal cauda was cut and kept in 0.5 mL of pre-warmed PBS (pH 7.3) having a drop of nigrosin stain. Fifty microliters of the homogenate were placed on a slide at 37 °C for microscopic observation at 40X. At least 10 fields were observed, and almost 100 sperm per sample were analysed for sperm motility. The samples were analysed in triplicate, and the averaged as the motility of total sperm (Halvaei *et al.*, 2012a).

Sperm count

The epididymal caudal was taken in 1 ml solution of physiological saline. The tissues were crushed with scissors gently and kept at 37 °C for 15 min. Nigrosin (2-3 drops) was added to the tissues. About 10 µL of the homogenate was placed on a warm slide. At least 10 fields were properly observed for spermatozoa count using a light microscope at 40X.

Assessment of sperm viability

The eosin-nigrosin staining procedure was performed to assess sperm viability. The eosin-nigrosin dye (25 µL) was mixed with a semen sample. A drop of this mixture was placed on the slide, and a smear was prepared and dried at room temperature. The prepared

slides were observed under a microscope at 40X. The live spermatozoa remained white (unstained), while the dead spermatozoa were stained red. The percentage of live and dead spermatozoa were calculated as previously described by Halvaei *et al.,* (2012b).

Sperm abnormality

After the dissection procedure, epididymal cauda region was cut and put into 0.5 ml PBS (pH 7.3) pre-warmed at (37 ^{0} C). Sperm smear was put on a slide and air-dried overnight for analysis of morphological abnormalities of sperms. These slides were properly stained with 1 % eosin-Y/5 % nigrosine and further examined under a light microscope at 40X for abnormalities i.e., abnormal tails, hookless, bicephalic, amorphous, and coiled (Organization, 2010).

Hormonal analysis

Intratesticular T and plasma T concentrations were quantified using ELISA kits (Cat # BC-1115, Bio Check Inc., USA) and LH, FSH and cortisol were determined by ELISA kits; for LH, FSH (Reddot Biotech Inc. Canada) and cortisol (Cal biotech, Inc. USA) according to the instructions in the kit.

Analysis of brain monoamines

Brain levels of norepinephrine (NE), serotonin (5-HT) and the 5-HT metabolite 5-hydoxyindole-3-acetic acid (5-HIAA), dopamine (DA) and the DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were analyzed by using HPLC-EC previously done by Øverli et al. (1999) and Ullah et al., (2019) with some slight modifications and changes.

Tissue histopathology

Take testis and epididymis tissue samples, then fixed all the samples for 48 hrs in 10% formalin. Used the ascending concentrations of alcohol at room temperature to carry out dehydration and shifting to xylene. After clearing, tissues were fixed in paraffin wax and five to seven µm thick sections were cut using a microtome (Thermo Fisher Scientific, UK). Sections were transferred to albumenized slides that were preheated to 37 °C. For complete stretching of tissues and removal of bubbles, slides were incubated overnight at 58 °C. Tissues were rehydrated in descending concentrations of alcohol, stained with hematoxylin-eosin stain, and covered with a coverslip. The ready slides were monitored under a Leica microscope (New York, USA) equipped with a digital camera (Canon, Japan) at 40× magnification. The percentage area covered by seminiferous tubules was calculated using ImageJ software as previously described by (Jensen, 2013). The area in percentage was calculated through the given formula

$$\% As = As \times 100/T$$

Where; As is the area covered by seminiferous tubules and T is the total area of the field. The % age of the mean area was calculated between control and treated groups for comparison.

Statistical analysis

All statistical analyses were carried out by using easy nova (Arnhold, 2013) and lme4 (Bates *et al.*, 2014) package of R 3.2.5. The overall results were mentioned as mean ± SEM. *Shapiro-Wilk*, *Levene's* and *Tukey1-dF test,* were used for the assumption of normality, homogeneity of variances, and additivity of the statistical model. The effects of treatment with different furan concentrations were analyzed using the ANOVA ea1 test command of the R program with completely randomized design followed by post hoc *Tukey's HSD* (R Development Core Team, 2016). The values of $p<0.05$ were considered significant statistically.

RESULT

Effect of *in vivo* furan exposure on body weight and reproductive organ weight of rats.

A non-significant (P=0.95) change was observed in animal weight gain among all groups (Table 4). However, Furan exposure also did not alter the weights of the testis, epididymis, seminal vesicles, and prostate gland (Table 4).

Table 4. Effect of *in vivo* furan exposure on body weight and reproductive organ weight of rats.

Parameters	Furan (mg kg^{-1})					
	Control	5	10	20	40	P and F value
Body weight gain (g)	54.31 ± 2.15	50±4.59	51.6±2.71	51.6±4.11	51.2±4.84	P=0.95, F=0.16
Right testis weight (g)	1.37±0.04	1.31±0.10	1.24±0.04	1.33±0.08	1.32±0.09	P=0.83, F=0.35
Left testis weight (g)	1.30±0.11	1.29±0.14	1.21±0.09	1.13±0.08	1.12±0.09	P=0.63, F=0.64
Right epididymis weight (g)	0.68±0.05	0.60±0.09	0.62±0.05	0.69±0.05	0.75±0.07	P=0.46, F=0.93
Left epididymis weight (g)	0.63±0.07	0.45±0.08	0.59±0.02	0.73±0.04	0.69±0.10	P=0.11, F=2.13
Prostate (g)	0.63±0.05	0.57±0.09	0.44±0.07	0.47±0.08	0.51±0.09	P=0.47, F=0.91
Seminal vesicle (g)	0.73±0.24	0.59±0.08	0.51±0.04	0.38±0.02	0.38±0.04	P=0.21, F=1.59

P and F values in the rows were obtained from ANOVA with completely randomized simple designs, followed by Tukey's post hoc analysis, which shows a pairwise comparison of control and furan-treated groups. Mean values with different superscripts show significant differences (P<0.05) in the columns when comparing control with other treatment groups.

Effect of in vivo furan exposure on biochemical parameters of rat testis

Data of total protein content, ROS and antioxidant enzyme activity of the control and treatment groups are presented in Table 5. After 28 days of the furan treatment, the values of SOD and POD significantly ($P<0.05$) decreased in higher dose treatment groups than in the control group. A decrease in CAT activity was also seen in higher dose treatment groups. However, a minor increase in LPO activity was observed in the high-dose treatment groups, but this increase was not statistically significant (Table 5, Fig 7). The activity of ROS was significantly ($P<0.05$) increased in higher dose groups, i.e., 20 mg kg^{-1} and 40 mg kg^{-1} treatment groups. A significant ($P= 0.001$) decrease in protein content was observed in high-dose treatment groups, i.e., 10 mg kg^{-1}, 20 mg kg^{-1} and 40 mg kg^{-1} after 28 days of furan exposure (Table 5, Fig 7). A significant increase ($P=0.001$) in triglyceride, cholesterol, and LDL levels, but a decrease in HDL ($P <0.001$) was appeared in high-dose groups (10 mg kg^{-1}, 20 mg kg^{-1} and 40 mg kg^{-1}) versus the control group (Table 5, Fig 8).

Effect of *in vivo* furan exposure on epididymal sperm parameters of rats.

Furan caused a significant decrease in sperm motility ($P=0.007$), sperm count ($P < 0.001$), and sperm viability ($P < 0.001$) in the high-dose furan-treated groups when compared with the control group (Table 6).

Table 5. Effect of *in vivo* furan exposure on biochemical parameters of rats.

Parameters	Furan (mg kg^{-1})					Statistics
	Control	5	10	20	40	P &F value
SOD (Umg^{-1} protein)	23.35±2.15^{a}	20.09±2.36^{ab}	20.48±1.48^{a}	15.58±1.60^{ab}	12.16±1.64^{b}	P=0.003, F=5.57
POD (n mole min^{-1}mg^{-1} protein)	15.73±1.79^{a}	14.40±2.97^{ab}	9.14±1.52^{bc}	7.86±1.01^{c}	11.46±1.87^{ac}	P=0.04, F=2.96
CAT (Umg^{-1} protein)	10.09±0.99^{a}	7.08±1.02^{a}	6.77±2.12^{a}	6.60±0.91^{a}	5.85±0.92^{a}	P=0.35, F=1.16
LPO (nM TBARmin^{-1}mg^{-1})	1.81±0.35^{a}	1.94±0.41^{a}	2.04±0.44^{a}	7.65±1.06^{a}	6.34±1.81^{a}	P=0.54, F=0.79
Total ROS (Umg^{-1} tissue)	1.38±0.07^{b}	2.02±0.09^{b}	2.41±0.04^{ab}	3.07±0.15^{ab}	3.33±0.06^{a}	P=0.05, F=2.86
Protein (mg 0.5g^{-1})	60.78±2.66^{a}	49.32±1.89^{b}	43.23±2.53^{bc}	42.65±2.34^{bc}	36.28±2.85^{c}	P=0.001, F=13.8
Cholesterol (mg dl^{-1})	46.2±2.58^{d}	48±1.59^{cd}	64±2.22^{b}	58.4±2.02^{bc}	72.2±1.77^{a}	P=0.001, F=29.8
Triglyceride (mg dl^{-1})	76.71±1.60^{e}	93.2±2.58^{d}	104.8±1.74^{c}	120.8±2.46^{b}	136±0.95^{a}	P=0.001,,F=139.51
HDL (mgdl^{-1})	60.16±1.94^{a}	40.51±2.78^{b}	37.2±3.34b^{b}	35.7±2.29^{b}	34±0.70^{b}	P<0.001, F=19.97
LDL (mg dl^{-1})	34.6±3.86^{c}	51.8±2.99^{b}	51.2±2.22^{b}	59.2±1.68^{b}	70.8±1.32^{a}	P=0.001, F=26.15

P and F values in the rows were obtained from ANOVA with completely randomized designs, followed by Tukey's post hoc analysis, which shows a pairwise comparison of control and furan-treated groups. Mean values with different superscripts show significant differences (P<0.05) in the rows when comparing control with other treatment groups

[a] Values are presented as Mean ± (SEM), [b] Significance at P<0.05 vs control, [c] Significance at P<0.001 vs control

[d] Significance at P<0.01 vs control

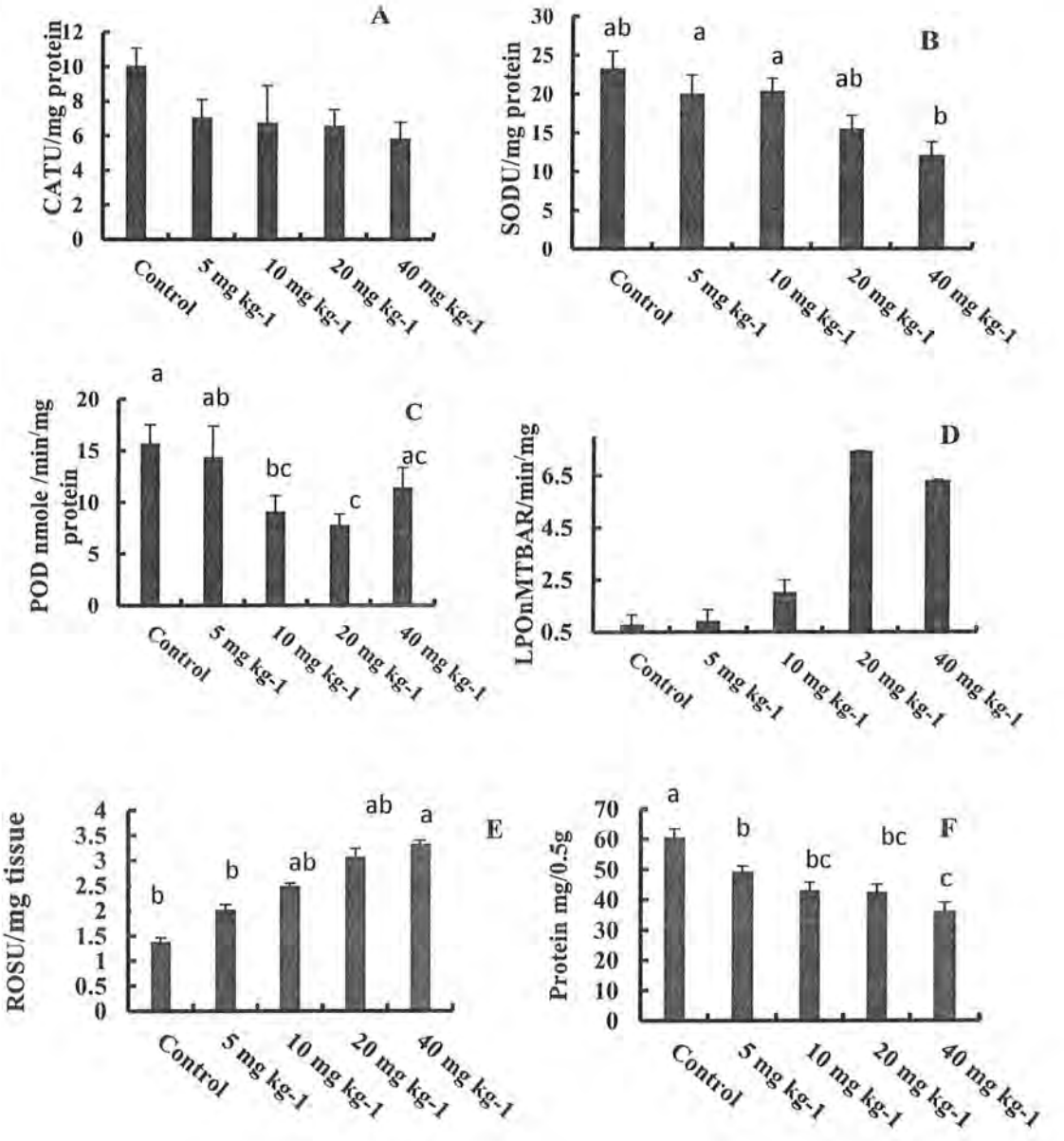


Fig 7. Effect of *in vivo* furan exposure on biochemical parameters of rat testis treated with different doses of furan, CAT(A) POD(B) SOD(C) ROS(D) TBARS (E) and Protein(F).

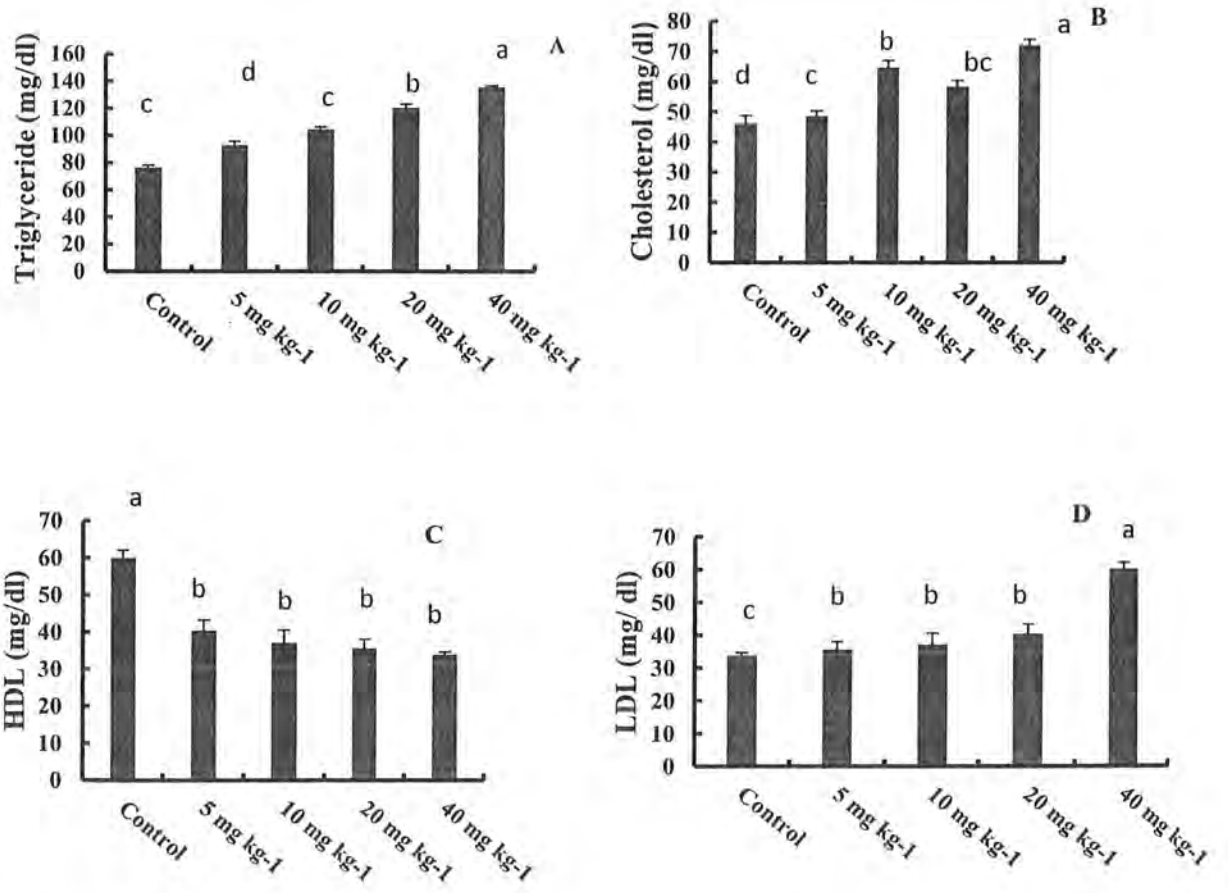


Fig 8. Effect of *in vivo* furan exposure on biochemical parameters of rat testis treated with different doses of furan, Triglycerides (A) Cholesterol (B) HDL (C) LDL (D).

Table 6. Effect of *in vivo* furan exposure on epididymal sperm parameters of rats.

	Furan (mg kg $^{-1}$)					
	Control	**5**	**10**	**20**	**40**	**Statistics**
Sperm count (×10^6/gm of cauda)	176±5.09^a	158±3.11^{ab}	150.6±3.59^{bc}	129.3±5.80^{cd}	123.3±6.23^d	P=0.001, F=19.78
Sperm viability %	84.33±3.82^a	74.66±2.65^{ab}	72.33±2.71^{ab}	60.66±2.37^b	65.33±3.82^b	P=0.002, F=8.69
Sperm motility%	67.66±2.37^a	56.66±4.49^{ab}	55±5.88^{ab}	46±6.79 ^{b}	43.6±1.89^b	P=0.02, F=4.70

P and F values in the rows were obtained from ANOVA with completely randomized designs, followed by Tukey's post hoc analysis, which shows a pairwise comparison of control and furan-treated groups. Mean values with different superscripts show significant differences ($P<0.05$) in the rows when comparing control with other treatment groups.

Effect of *in vivo* furan exposure on the hormonal concentration of rats.

Intratesticular and plasma T concentrations in control and furan-treated groups are shown in Table 7. Plasma T concentration was significantly (P=0.04) lowered in the high-dose (40 mg kg^{-1}) treatment group. Similarly, a significant (P=0.02) reduction in intratesticular T concentration was also seen in the treatment group when compared with that in the control group, while in the other treatment groups, no significant differences were observed. Similarly, significant reductions in plasma LH (P=0.004) were observed in the groups receiving the two highest furan doses (Table 7). Also, a reduction in plasma FSH concentration was observed, but this reduction was not statistically significant. Cortisol concentrations were significantly elevated in the 20 and 40 mg kg^{-1} (P<0.001 and P=0.005) treated group; Table 7).

Table 7. Effect of *in vivo* furan exposure on the hormonal concentration of rats.

	Furan (mg kg^{-1})					
	Control	**5**	**10**	**20**	**40**	**Statistics**
Plasma T (ng ml^{-1})	4.56 ± 0.46^{a}	3.47 ± 0.02^{ab}	3.09 ± 0.46^{ab}	3.39 ± 0.19^{ab}	2.18 ± 0.26^{b}	P=0.05, F=3.40
Intra-testicular T (ng g^{1}tissue)	26.27 ± 2.82^{a}	22.83 ± 2.54^{ab}	18.61 ± 2.96^{bc}	16.88 ± 1.37^{bc}	14.61 ± 1.30^{c}	P=0.02, F=7.57
LH (ng ml^{-1})	1.50 ± 0.10^{a}	1.47 ± 0.08^{a}	1.32 ± 0.06^{ab}	1.22 ± 0.07^{ab}	1.13 ± 0.04^{b}	P=0.004, F=4.31
FSH (ml Uml^{-1})	0.87 ± 0.03^{a}	0.84 ± 0.14^{a}	0.75 ± 0.10^{a}	0.69 ± 0.15^{a}	0.51 ± 0.03^{a}	P=0.13, F=1.84
Cortisol (ng ml^{-1})	42.83 ± 1.46^{d}	45.49 ± 1.48^{cd}	52.86 ± 2.69^{c}	61.12 ± 1.81^{b}	69.02 ± 1.79^{a}	P<0.001, F=32.76

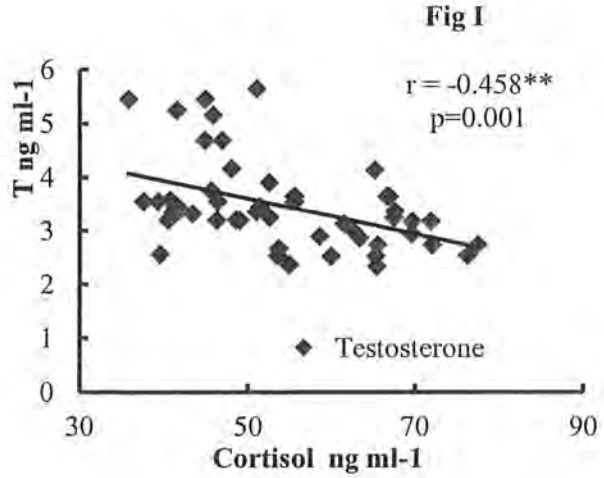
P and F values in the rows were obtained from ANOVA with completely randomized designs, followed by Tukey's post hoc analysis, which shows a pairwise comparison of control and furan-treated groups. Mean values with different superscripts show significant differences (P<0.05) in the rows when comparing control with other treatment groups.

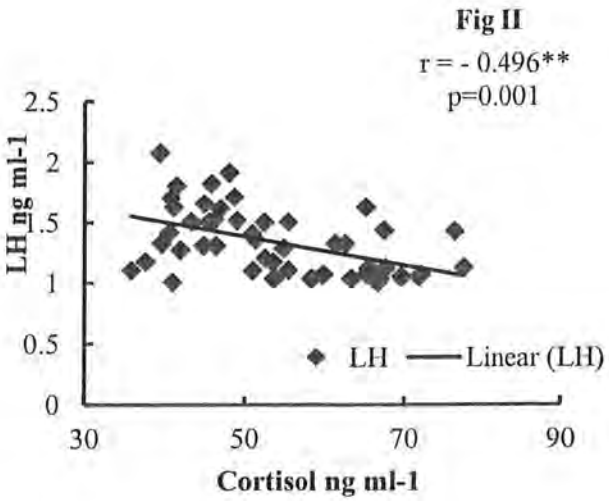
Develop relationship between plasma cortisol concentration and plasma T, LH and FSH hormones, a bivariate correlation was performed (Table 8, Fig 9). Results showed that plasma cortisol concentration has a significant negative correlation with plasma T (r= -0.458, P=0.001), LH (r= - 0.496, P=0.001) and negative non-significant correlation with FSH (r= -0.185, P=0.2). Moreover, a direct positive correlation was recorded among T and LH (r= 0.404, P=0.004); and T and FSH (r= 0.14, P=0.33). A positive yet not significant correlation appeared between LH and FSH (r=0.169; P=0.2) (Table 8, Fig 9).

Table 8. Summary of the relationship of serum cortisol with serum T, LH and FSH in adult rats' after exposure to furan (0, 5, 10, 20 and 40 mg kg^{-1}) for 28 days.

	Correlations			
	Cortisol (ng ml^{-1})	**T (ng ml^{-1})**	**LH (ng ml^{-1})**	**FSH ($mlUml^{-1}$)**
Cortisol (ng ml^{-1})	r =1			
T (ng ml^{-1})	r = -0.458**	r = 1		
	P= 0.001			
LH (ng ml^{-1})	r = - 0.496**	r = 0.404**	r = 1	
	P=0.001	P=0.004		
FSH ($mlUml^{-1}$)	r = -0.184*	r = 0.14	r = 0.169*	r = 1
	P=0.2	P=0.33	P=0.241	

Pearson's correlation and sets significant relation; Pearson's correlation is shown with r whenever significant by p-value.





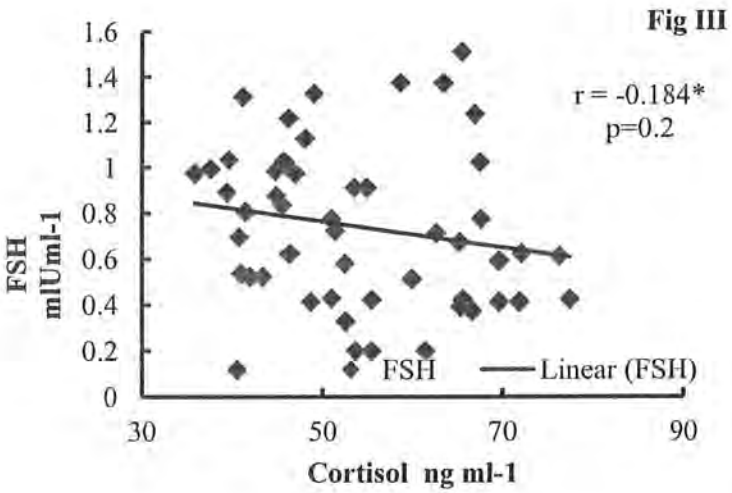


Fig 9. Correlation of serum cortisol with serum T (I) LH (II) FSH (III) in rats after exposure to furan (0, 5, 10, 20 and 40 mg kg^{-1}). Pearson's correlation r and p values are given.

Effect of *in vivo* furan exposure on brain monoamines levels of rat brain hypothalamus tissues.

The effect of subchronic exposure of furan on the serotonergic and dopaminergic activity of brain hypothalamus is shown in Table 9. A post hoc Tuckey test revealed a significant effect of furan exposure on serotonergic (5HIAA/5HT), dopaminergic (DOPAC/DA and HVA/DA) and norepinephrine NE activities in the hypothalamus. A significant ($P<0.001$) increase in 5HT/5HIAA ratios were observed in high doses 20 and 40 mg kg^{-1} groups of furan treatment in the brain hypothalamus region. Similarly, the alterations in dopaminergic (DOPAC/DA and HVA/DA) levels were observed in dose-dependent manners. A significant decreased in DOPAC/DA ($P=0.04$) and HVA/DA ($P= 0.02$) levels were observed in the hypothalamus brain region. After furan exposure, a significant ($P=0.01$) elevation was recorded in NE level compared to the control group. The level of brain monoamines showed +ve correlation with cortisol group.

Table 9. Effect of *in vivo* furan exposure on brain monoamines levels of rat brain hypothalamus tissues.

	Furan mg k g^{-1}					
	Control	**5**	**10**	**20**	**40**	**Statistics**
5HIAA /5HT ratio	0.53 ± 0.03^{c}	0.53 ± 0.02^{c}	0.58 ± 0.03^{bc}	0.70 ± 0.03^{a} b	0.83 ± 0.05^{a}	$P<0.001$ $F=12.50$
DOPAC / DA ratio	0.20 ± 0.01^{a}	0.21 ± 0.01^{a}	0.22 ± 0.01^{a}	0.27 ± 0.02^{a}	0.32 ± 0.07^{a}	$P=0.04$ $F=2.58$
HVA / DA ratio	0.35 ± 0.02^{b}	0.39 ± 0.03^{ab}	0.42 ± 0.03^{ab}	0.42 ± 0.04^{a} b	0.55 ± 0.06^{a}	$P=0.02$ $F=3.09$
NE (ng g^{-1})	236 ± 9.72^{a}	244 ± 10.22^{a} b	253 ± 12.32^{a} b	270 ± 11.73 b	292 ± 11.60 b	$P=0.01$ $F=3.68$

P and F values in the rows were obtained from ANOVA with completely randomized designs, followed by Tukey's post hoc analysis, which shows a pairwise comparison of control and furan treated groups. Mean values with different superscripts show significant differences ($P<0.05$) in the rows when comparing control with other treatment groups.

To investigate the relationship between plasma cortisol concentration and brain serotonergic and dopaminergic activity we correlated these concentrations in a pairwise fashion (Table 10, Fig 10). Results indicated that plasma cortisol concentration is positively correlated with serotonergic activity of brain (r=0.55, P= 0.001**) and dopaminergic (DOPAC/DA ratio) (r=-0.35, P=0.01), (HVA/DA ratio) (r =0.25, P=0.07), and with NE (r =0.41, P=0.002). While the relationship between all brain monoamines is positively correlated with each other. Finally, a positive correlation appeared between the level of plasma cortisol versus monoamines of the brain hypothalamus region. (Table 10; Fig 10).

Table 10. Correlations of serum cortisol and brain monoamines (5HIAA/5HT, DOPAC/DA, HVA/DA and NE) concentrations after furan exposure (0, 5, 10, 20 and 40 mg kg^{-1}).

	Correlations				
	Cortisol (ng ml^{-1})	**5HIAA/5HT ratio**	**DOPAC/DA ratio**	**HVA/DA ratio**	**NE (ng g^{-1})**
Cortisol (ng ml^{-1})	r =1				
5HIAA/5HT ratio	r=0.55 P= 0.001**	r =1			
DOPAC/DA ratio	r = 0.35 P=0.01	r = 0.093 P=0.520	r =1		
HVA/DA ratio	r =0.25 P=0.07	r=0.196 P=0.17	r=0.477 P=0.0004**	r =1	
NE (ng g^{-1})	r =0.41 P=0.002	r= 0.43 P= 0.001**	r=0.125 P=0.386	r=0.003 P=0.981	r =1

Pearson's correlation and sets significant relation; Pearson's correlation is shown with r whenever significant by p-value.

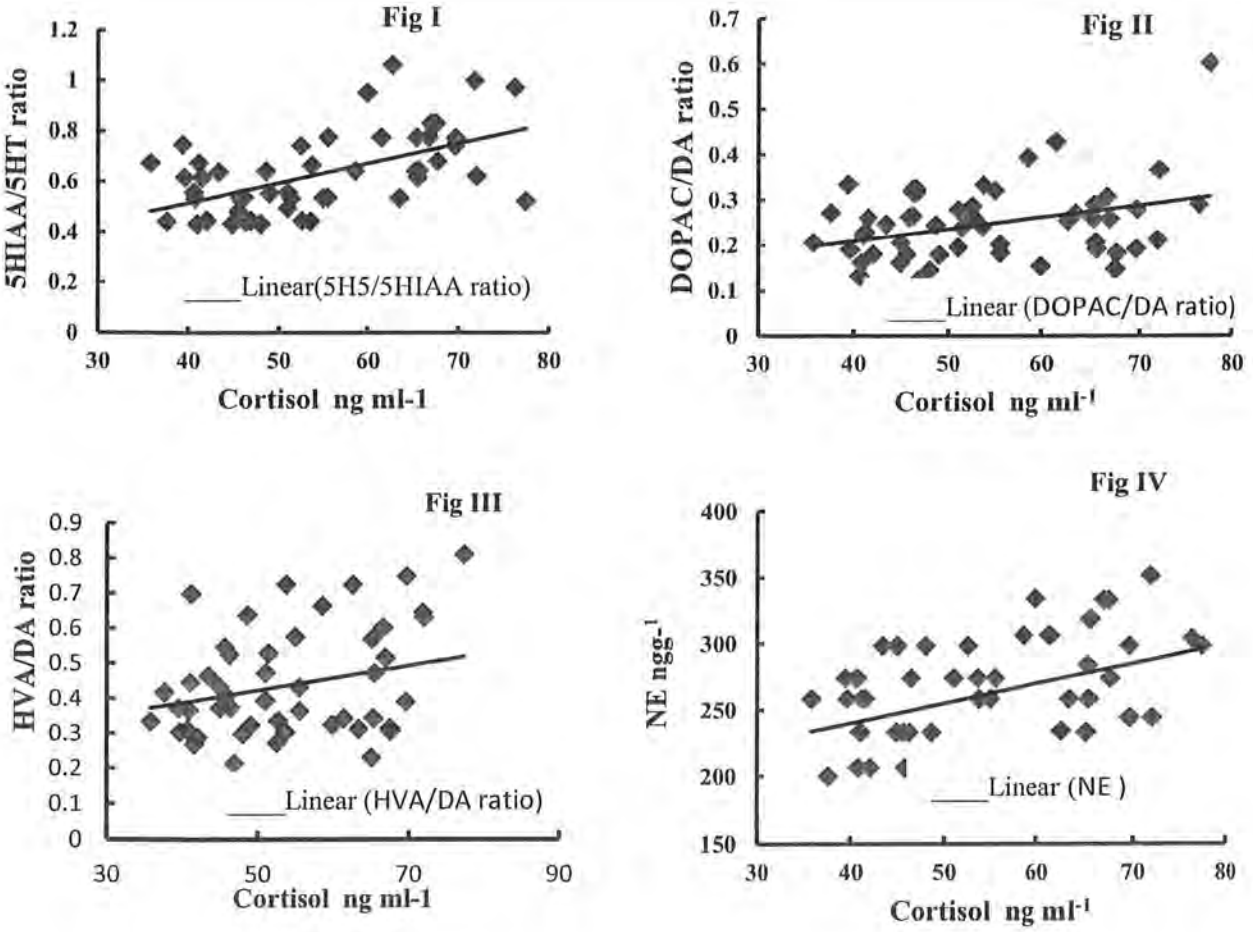


Fig 10. Brain monoaminergic activity 5HIAA/5HT ratio (I) DOPAC / DA ratio (II) HVA / DA ratio (III) and NE (IV) as a function of plasma Cortisol concentration in adult rats after exposure to furan. Pearson's correlation coefficient (r) and p values are provided in tables.

Histopathological analysis

Testis

Different parameters of histopathological findings of the testicular and epididymal tissues are mentioned in (Table 11; Fig 11). Testicular tubular and testicular lumen diameters showed significant ($P < 0.001$) difference when compared with the control group. High-dose (20 mg kg^{-1} & 40 mg kg^{-1}) groups showed significant ($P=0.001$) decrease in tubular diameter and significant ($P=0.001$) increase in lumen diameter of the testis. However, epithelial height was significantly ($P=0.05$) reduced in the high-dose treatment groups compared to the control group. However, a significant ($P=0.05$) decrease was observed at tunica albuginea height in the 40 mg kg^{-1} treatment group compared to the non-treated group (Table 11; Fig 11).

Caput epididymis

Histopathology of the epididymal caput region showed no visible changes in caput epithelial heights and lumen diameters (Fig. 12). However, the caput tubular diameter showed significant ($P<0.001$) difference in the high-dose 20 and 40 mg kg^{-1} groups compared to control group. Epithelium % and lumen % also showed some differences between control and treated groups. However, all these differences were not highly significant compared to control (Table 11; Fig. 12).

Cauda epididymis

The ductular and lumen diameter of cauda showed nonsignificantly ($P = 0.93$) changes. Epithelial height of the caudal epididymis was significantly ($P=0.005$) reduced in the high-dose groups when compared with that in the control group. Epithelium percentage and lumen percentage also showed some differences between control and treatment groups, but this difference was not significant (Table 11; Fig 13).

Table 11. Effect of *in vivo* furan exposure on histopathology of rat testicular and epididymis (caput and caudal) tissues.

Parameters		Furan (mg kg^{-1})					Statistics
		Control	5	10	20	40	P and F Values
Testis	Seminiferous tubule diameter (µm)	238.46±6.82^{a}	236.6±6.91^{a}	212.31±6.48^{b}	178.95±7.70^{c}	163.34±5.79^{c}	P=0.001, F=24.99
	Tubular lumen diameter (µm)	26.65±1.14^{c}	27.91±0.75^{c}	32.39±0.74^{b}	37.57±0.83^{a}	36.09±1.10^{ab}	P=0.001, F=24.9
	Epithelial height (µm)	92.67±5.42^{a}	89.47±4.33^{a}	88.68±4.49^{ab}	81.82±4.51^{ab}	75.95±2.62^{b}	P=0.05, F=2.34
	Tunica albugenia height (µm)	23.92±1.53^{a}	20.79±1.39^{ab}	20.65±1.11^{ab}	18.10±1.34^{ab}	16.09±0.84^{b}	P=0.04, F=2 .63
Epididymis caput region	Tubule diameter (µm)	333.07±8.26^{a}	317.46±7.83^{ab}	306.30±7.51^{b}	272.85±6.23^{c}	250.35±6.94^{d}	P=0.001, F=20.0
	Lumen diameter (µm)	240.84±7.65^{a}	247.66±9.69^{a}	257.02±7.30^{a}	257.02±7.30^{a}	257.02±7.30^{a}	P=0.50, F=0.83
	Epithelial height (µm)	32.89±1.143^{a}	29.77±1.43^{ac}	30.84±1.48^{ab}	27.76±1.43^{bc}	26.20±1.56^{c}	P=0.01, F=3.25
	Epithelium %	41.12±1.42^{a}	39.12±1.67^{a}	38.55±1.85^{a}	36.73±1.90^{a}	35.171±1.85^{a}	P=0.16, F=1.63
	Lumen%	58.87±1.42^{b}	62.77±1.79^{ab}	61.44±1.85^{ab}	65.29±1.79^{ab}	67.23±1.95^{a}	P=0.01, F=3.259
Epididymis caudal region	Tubule diameter (µm)	462.73±7.64^{a}	460.34±10.16^{a}	457.82±8.46^{a}	454.88±9.05^{a}	450.73±9.66^{a}	P=0.83, F=0.36
	Lumen diameter (µm)	417.76±10.4^{a}	415.25±9.2^{a}	413.34±7.47^{a}	408.19±6.4^{a}	410.46±8.75^{a}	P=0.93, F=0.199
	Epithelial height (µm)	35.80±1.15^{a}	34.84±1.42^{a}	29.11±1.5^{ab}	31.13±1.55^{b}	28.76±1.49^{b}	P=0.01, F=5.166
	Epithelium%	44.75±1.44^{a}	43.55±1.77^{ab}	36.39±1.8^{ac}	39.1±1.94^{bc}	35.96±1.87^{c}	P=0.001, F=4.16
	Lumen%	55.24±1.44^{a}	56.44±1.77^{a}	61.29±2.02^{a}	58.55±2.09^{a}	61.15±2.11^{a}	P=0.08, F=2.082

P and F values in the rows were obtained from ANOVA with completely randomized designs, followed by Tukey's post hoc analysis, which shows a pairwise comparison of control and furan-treated groups. Mean values with different superscripts show a significant difference (P<0.05) in the rows when comparing control with the other treatment groups. [a] Values are presented as Mean ± (SEM), [b] Significance at *P<0.05* verses control, [c] Significance at *P<0.001* verses control, [d] Significance at *P<0.01* versus control

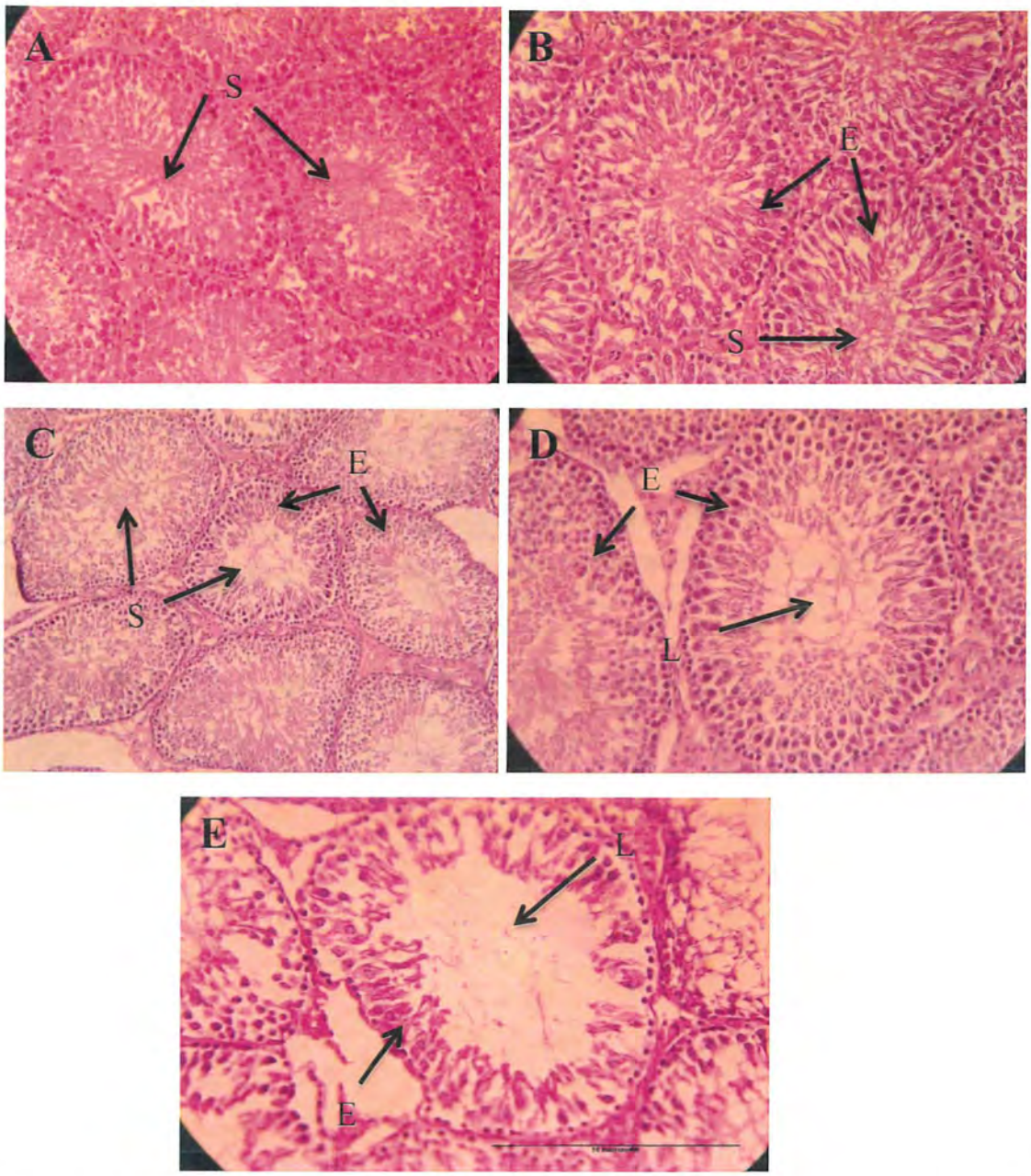


Fig 11. Photomicrograph of the seminiferous tubules of male rats receiving doses of furan. (A) Control; showing regularly arranged tubules, lumen filled with spermatids with normal germ cells (B) 5 mg kg^{-1} group; showing the normal process of spermatogenesis, lumen filled with mature spermatozoa, (C) 10 mg kg^{-1} group; normal morphology with slightly widened lumen (D) 20 mg kg^{-1} group; showing increased lumen diameter and amplified epithelial height, tubules with interstitial spaces are present, (E) 40 mg kg^{-1} group; thin degenerated epithelium with an empty lumen. Magnification x40. Lumen (L), Epithelium (E), Spermatozoa (S).

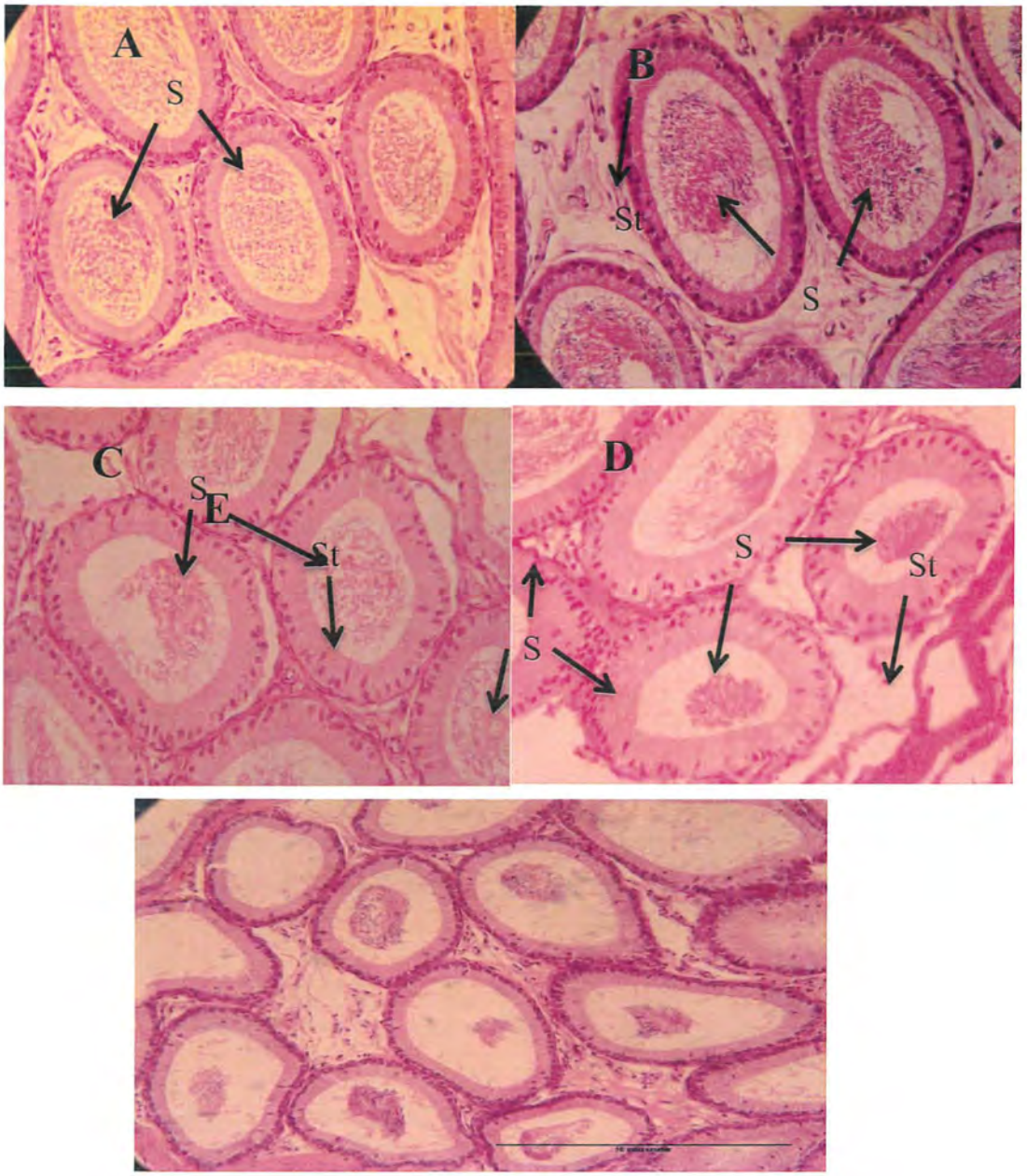


Fig 12. Photomicrograph of cross-section of caput epididymis (H&E, 40X) of male rats receiving doses of furan showing (A) Control; exhibiting normal morphology of caput epididymis; compactly arranged tubules with thick epithelium, lumen filled with mature sperms, (B) 5 mg kg^{-1} group; showing normal morphology like control. (C) 10 mg kg^{-1} group; showing slight changes in morphology of tubules, surrounded by stroma. (D) 20 mg kg^{-1} group; lumen has very a smaller number of spermatozoa. (E) 40 mg kg^{-1} group; showing a further increase in epithelial height and a slight increase in lumen sperm concentration. Spermatozoa (S), Stroma (St).

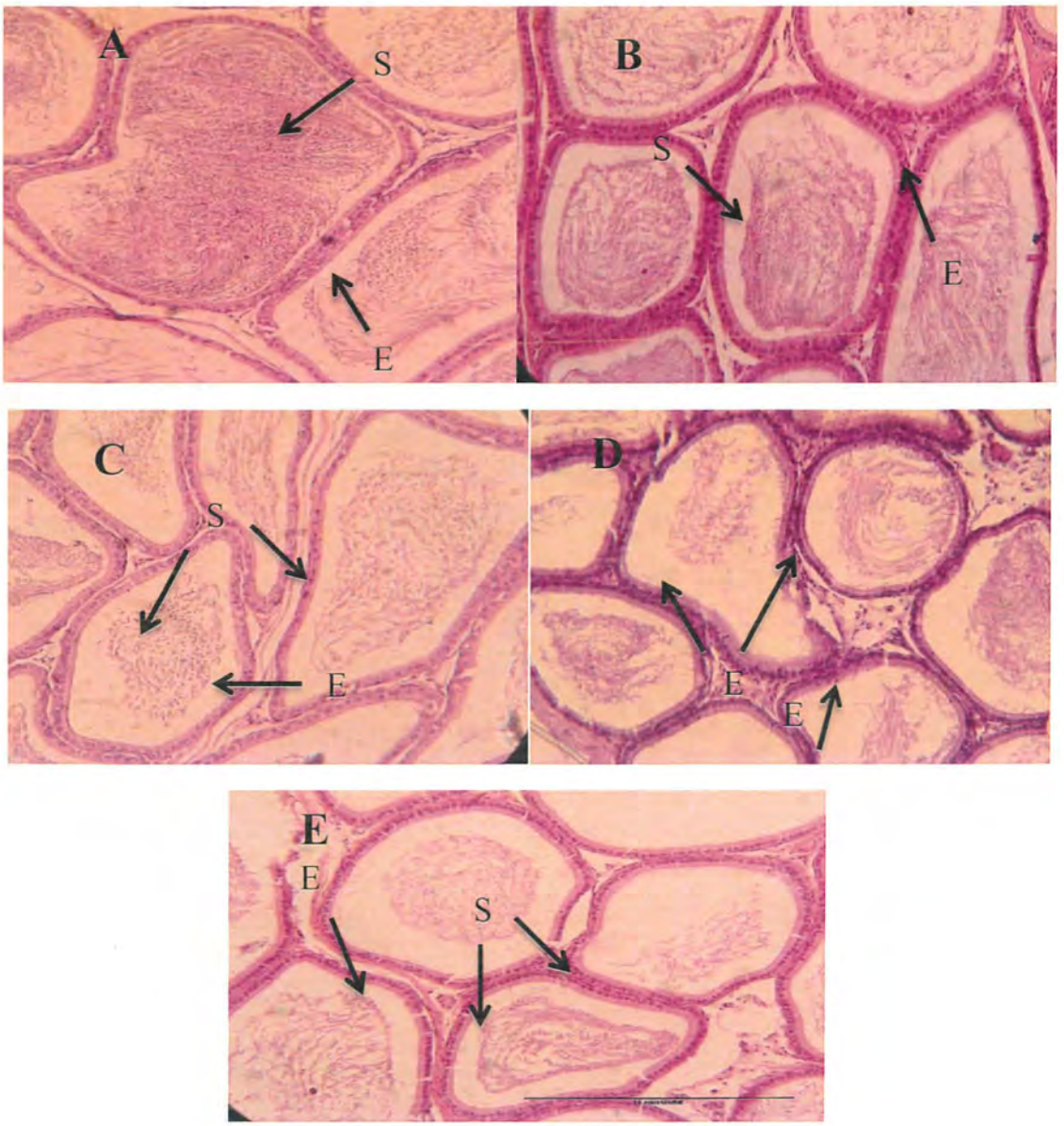


Fig 13. Photomicrograph of cauda epididymis of adult male rats receiving doses of furan (H&E, 40X) from (A) control group; with normal morphology of cauda epididymal cells having thin pseudostratified epithelium of the lumen heavily filled with spermatozoa, (B) 5 mg kg^{-1} group; with thin epithelium lined with stereocilia, (C) 10 mg kg^{-1} group; showing normal spermatozoa in the lumen (D) 20 mg kg^{-1} group; showing an increase in the pseudostratified epithelium and very little concentration of spermatozoa, (E) 40 mg kg^{-1} group; further reduction in spermatozoa and increased epithelial height. Epithelium (E), Spermatozoa (S).

DISCUSSION

Furan is formed from several precursor molecules naturally present in food during various steps of processing, which might involve oxidation or Maillard reaction (Limacher *et al.*, 2007; Bolger, 2009; Limacher *et al.*, 2008). It has been previously reported that furan is a toxic agent that causes various harmful effects on biological systems of living organisms(Gill *et al.*, 2010; Jackson *et al.*, 2014; Webster *et al.*, 2014; Pandir, 2015; Uçar and Pandir, 2017). Previous literature on furan reported that its exposure causes reproductive impairment by disturbing spermatogenesis, thereby leading to apoptosis in germ cell lining and Leydig cells. Thus, on a global scale, furan is of high concern because of its harmful effects on animals and humans (Hamadeh *et al.*, 2004). The current study was designed to find out the effect of furan on brain monoamines levels and reproductive functions by an *in vivo* experiment. Similarly, *in vivo,* sub-chronic exposure study results indicated that higher doses of furan induced more reproductive toxicity than lower doses. High doses of furan, i.e., 20 & 40 mg kg^{-1} produce more ROS and oxidative damage in testicular tissues by reducing the diameter of seminiferous tubular lumen, epithelial height and reducing sperm number in the epididymis lumen. All these changes may be due to high ROS production. Oxygen-containing molecules that have free, unpaired electrons and are highly reactive. Some nonradical molecules also act as a ROS. Oxidative phosphorylation occurs in mitochondria, which results in the production of ROS (Devasagayam *et al.*, 2004). The high ROS production imparts damaging effects on protein, lipids, and DNA (Radak *et al.*, 1999). The antioxidant enzyme is activated in response to the production of ROS in the cells, which reduce the level (Kaul and Forman, 2000). The decrease in the antioxidant enzyme levels devastates the cell's sensitivity to harbour oxidative stress, hence compromising its ability to detoxify the effects of ROS (Kaul and Forman, 2000; Pérez 2009). Previously, furan was found to be a cytotoxic, genotoxic, and an apoptosis inducer (Heppner and Schlatter, 2007; Karacaoğlu and Selmanoğlu, 2010). It has already been reported that oxidative stress degrades protein and lipids (Radak *et al.*, 1999). Lipids have important structural and functional roles in different body organs and cells (Rawi *et al.*, 2012) and are also important for maintaining body functions. Oxidative stress causes derangement in serum lipid concentration and disturbance in lipid profile also called

dyslipidemia (abnormal amount of triglycerides, cholesterol, and fat phospholipids) in the bloodstream (Martins *et al.*, 2018). In the current study, plasma cholesterol, LDL and triglyceride levels were elevated in furan-treated animals, while the HDL level was reduced. According to previous literature, furan and acrylamide administration causes an increase in plasma LDL, total cholesterol, triglycerides and decrease in HDL (Ghanayem *et al.*, 2010; Raju *et al.*, 2015). Previously, researchers have reported that the association of increased cholesterol and reduced HDL levels is linked to male infertility, low T level, and sperm abnormality (Shalaby *et al.*, 2004).

In current findings, a clear decrease was observed in sperm count. Similar observations were also stated by (Uzunhisarcikli *et al.*, 2007), where sperm count reduced as a result of the reduction in T, vel. Spermatozoa exposed to a high concentration of ROS resulted in reduced sperm viability and motility, leading to infertility (Aitken and Curry, 2011) as reproductive hormones control spermatogenesis and cell-to-cell interaction within the testis. In the present study, histopathological findings showed an increase in seminiferous tubular diameter and lumen diameter, while epithelial height declined in a dose-dependent manner. Seminiferous tubules with the hollow lumen and less interstitial space were observed in high-dose furan administration. These histopathological findings showed the endocrine-disrupting nature of furan. As reported in previous studies, that decline of seminiferous epithelium connected to the T reduction, and decreased T levels further reduce the number of germ cells in stages VII to VIII (Kumar *et al.*, 2006; Karacaoğlu and Selmanoğlu, 2010).

In the current investigation, plasma and T levels were lower in the furan treated groups. Likewise, plasma LH as well as FSH concentrations were also compact in the treated animals. The gonadotropins perform the main part in the spermatogenesis and during steroidogenesis process. At the same time, oxidative stress and T production has been interconnected and T synthesis is decreased by ROS (García *et al.*, 2012). Reproductive hormones control the spermatogenesis and cellular communication within the testis. The reduction of LH may be resulted due to reduction of T while. It has been previously reported that increased cortisol and oxidative stress decreased reproductive

hormonal level. Antioxidant enzyme depletion cause alteration spermatogenesis, and sperm maturation. A decrease in the T production in adulthood is a recurrent finding in studies exposing rats to heat-induced food toxicants (Salian *et al.*, 20090; El-Akabawy and El-Sherif, 2016).

The increased cortisol secretion has been associated with a decreased production of sex steroids and GH in a multitude of studies (Burguera *et al.*, 1990; Björntorp, 1995; Chen *et al.*, 1997; Tsigos and Chrousos, 2002; Viau, 2002; Liening and Josephs, 2010). High doses of glucocorticoids inhibited testicular Leydig cell function in rats (BAMBINO and HSUEH, 1981). In humans, exposure to cortisol caused a marked decrease in T production (Cumming *et al.*, 1983). Glenn, (2011) also reported that cortisol also inhibits HPG axis activity at all levels. Similar findings were observed in the current investigation that cortisol was increased and the level of gonadotropin and T level decrease furan treated individuals. The seminiferous tubules epithelial height and number of spermatids are reduced indicating the capability of furan as an endocrine disruptor. Endocrine disruptors such as PAHs and PCBs, as well as lead and cadmium cause, elevated cortisol level in fish and mammals (Tort *et al.*, 1996; Tan *et al.*, 2007; Zimmer *et al.*, 2009; Sajjad *et al.*, 2018). The reduction in T concentration in current findings might be due to the anti-androgenic property of furan as reported by (Cooke *et al.*, 2014; Pandir, 2015). The relationships we observed between plasma hormone levels furthermore fit well with earlier studies reporting negative relationships between plasma cortisol vs. T (Wennink *et al.*, 1990). Indeed, many components of the gonadal axis are downregulated by plasma glucocorticoids, either by affecting hypothalamus and pituitary functions or by affecting the responsiveness of target tissues to gonadal hormones (Thakore and Dinan, 1994; Borges *et al.*, 1997). The HPA-axis is disrupted by both non-chemical (social stressors) and chemical factors reported by (Miller *et al.*, 2007; Cory-Slechta *et al.*, 2008). It has been previously observed that elevated cortisol cause energetic costs (Leal *et al.*, 2011) reproduction (Schreck, 2010; Fitzpatrick *et al.*, 2012) and neurogenesis (Sørensen *et al.*, 2011). The HPT and HPA axis, also play a significant role in reproductive performance i.e., transcriptional, receptors, hormones, or cellular "crosstalk".The HPA-axis also modulate the HPG-axis activity and vice versa (Dobson *et al.*, 2003).

Environmental toxicant and EDCs have been reported that exert neurotoxic effects that interrupt the synthesis, release and transportation of neurotransmitters (Dickerson and Gore, 2007; Rasier *et al.,* 2007). In the current investigation, increased 5HIAA/5HT, DOPAC/DA and HVA /DA and NE were observed among high treated groups. Similar findings were previously reported that EDCs and non-EDCs, induced additive or synergistic effects (Schug *et al.,* 2015). Ali et al (1983) suggested that acrylamide single and repeated doses resulted in the elevation of 5-hydroxy indoleacetic acid regions hypothalamus, hippocampus, striatum, frontal cortex, and brain stem. Previous studies indicated that Pb and Cd affect the monoaminergic mechanism during development CNS (Bressler and Goldstein, 1991; Antonio *et al.,* 1996; Mejia *et al.,* 1997; Antonio *et al.,* 1998; Antonio *et al.,* 1999; Antonio *et al.,* 2002). Another environmental toxicant and EDCs cadmium affect neurotransmitter on exposure (Lafuente *et al.,* 2001). Cd exposure affects DA, 5-HT, and NA, and the severity effect based on its route, dose, and exposure timing (Lafuente *et al.,* 2001; Lafuente *et al.,* 2003). Similarly, polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) also modulate brain serotonergic activity (Gesto *et al.,* 2006; Clotfelter *et al.,* 2010; Rahman *et al.,* 2011). It has been also observed that PCB affect the brain the serotonin concentrations (Boix and Cauli, 2012).

In the present study, elevated monoamines in response to chemical exposure and their +ve correlation with cortisol indicated that monoamines may be involved in the regulation HPA axis. Brain 5-HIAA/5-HT levels can be correlated with plasma cortisol concentrations, suggesting that brain 5-HT an important role in the regulation of HPI-axis (homologue to mammalian HPA axis) (Winberg *et al.,* 1997; Winberg and Lepage, 1998; Hoglund *et al.,* 2000) and hypothalamus–pituitary–adrenal (HPA) axes (Dinan, 1996; Heisler *et al.,* 2007). The 5HT is also involved in HPA axis regulation (Curtis and Patel, 2008). Serotonergic and dopaminergic system of the brain plays an important role in the regulation of the mechanism of stress (Winberg *et al.,* 2001; Bowman *et al.,* 2002; Larson *et al.,* 2003; Perreault *et al.,* 2003; Lepage *et al.,* 2005; Gesto *et al.,* 2013), intervened by a stressor (Schjolden *et al.,* 2006; Gesto *et al.,* 2008, 2009). The noradrenaline also considers to associated with the activation of CRF release, that further activate the mammalian HPA axis (Dunn *et al.*, 2004).

It has been previously reported that neurotransmitters are associated with neuroendocrine mechanism associated reproduction (Gallo, 1980; Nock and Feder, 1981). In the current study the brain monoamines that might be regulated by the neurotransmitters such as monoamine. Mammalians serotonin is performing various reproductive functions i.e., socio-sexual behaviours, secretion of GnRH, gonadotropin, gonadal maturation and reproductive endocrine signaling pathways, as well as serotonin, are closely associated (Prasad *et al.*, 2015). Invertebrates, the neuroendocrine regulate HPG axis, which highly conserved in vertebrates (Prasad *et al.*, 2015). 5HT is involved in the regulatory mechanism of gonadotropins because, it stimulates gonadotropin release through its interaction with receptors of 5HT in the median eminence (Spinell, 2000). The LH level in the current study in high doses of furan that might be the regulation of the elevated level of serotonin, Similar finding was also reported by Rubinow and Schmidt, (2006).

The maturation and development of spermatozoa and normal steroidogenesis is important for male fertility. The phenomenon of spermatogenesis and steroidogenesis in the testes are controlled by normal production and release of gonadotrophins LH and FSH (Naik et al., 2016). The appropriate levels of T are necessary to carry out normal functions of testes. The present findings showed that furan affected the normal function of leyding cells resulting in a significantly reduced production of T concentration in a dose-dependent manner. The reduction in testosterone concentrations is significantly contributed by the reduction in LH in male rats further encourage spermatogenic arrest and infertility.

CONCLUSION

Findings of the current study revealed that exposure to the high concentration of furan cause adverse effect on the brain monoaminergic activity, oxidative stress and exhibit structural aberration in reproductive organs that lead to alterations in reproductive functions of rats. Further, we suggest a detailed molecular-based study to investigate the exact mechanism and tissue-specific action of food-based contaminant furan in the reproductive organs and reproductive performance of rats.

Chapter No. 3

Possible effects of neonatal exposure to furan on sexual development of male rats.

ABSTRACT

The present study was investigated the reproductive toxicity of furan in male pups. Pups were separated into five groups on PND 0. The control group was injected with corn oil (50 µL), while the other four groups were injected with furan (1, 5, 10 and 20 mg kg^{-1}) from PND 1 to PND 10. In high dose groups, fertility was diminished, as evinced by minor DSP and epididymis sperm counts, and histological alterations in the testes. High dosage treatments (10 and 20 mg kg^{-1}) exhibited a significant drop in T, LH and GH while cortisol was increased. Contrarily, a significant upsurge in brain monoamines was observed in (10 and 20 mg kg^{-1}) treatments. The results propose that exposure to elevated dosages of furan at the neonatal stage caused organizational alterations in male rats, negotiating fertility.

INTRODUCTION

The recent decades have seen a rise in public attention to the adverse effects of chemicals on human health. Various environmental chemicals have been classified as endocrine-disrupting chemicals (EDCs) because of their ability to alter animal physiology by disrupting hormonal levels. Especially developmental stages are vulnerable since changes can become permanent when animals are exposed to EDCs during organ development (Richter *et al.,* 2007), changing the hormonal profile irreversibly (Lafuente *et al.,* 2003; Hinson and Raven, 2006; Liu *et al.,* 2010; Cooke *et al.,* 2014; Sajjad *et al.,* 2018). Such irreversible and long-lasting changes are termed "organizational effects" (Young, 1964; Arnold and Breedlove, 1985). On the other hand, EDCs may be less harmful after gonadal development is completed, as exemplified by the observation that men exposed to polychlorinated biphenyls at the age of 20 do not show a bias in the number of sons they conceive (del Rio Gomez *et al.,* 2002).

HPG axis and neuroendocrine control of reproduction are control by numerous aspects such as monoamine, amino acids, and peptides (Prasad *et al.*, 2015; Genazzani *et al.*, 2000; Zohar *et al.*, 2010). As previously reported that the brain controls the of the process of reproduction in many vertebrates and fish (Kah *et al.,* 1993). The brain is the master organ, which sends activatory and inhibitory signals for driving the sexual organs (Giuliano and Allard, 2001).In a brain (CNS system) several neurotransmitters are present. Neurotransmitters are functional in stressful conditions (Tsigos and Chrousos, 2002) and sexual functioning(Giuliano and Rampin, 2000; Heaton, 2000). Dysfunction of these monoamines has been related to a wide-ranging of central and peripheral disorders, and dysregulation of HPG and HPA axis (Sheikh *et al.,* 2007; Prasad *et al.,* 2015). Neurotoxicity caused by crossing the environmental and food toxicants through the blood-brain barrier (Leret *et al.,* 2003; Yadav *et al.,* 2010; Matsuda *et al.,* 2012).

In human infants, the main pathway of exposure to environmental toxicants is via the diet, either from breastfeeding or formula feeding (Lehmann *et al.,* 2018). It is well documented that environmental toxicants like bisphenol, arsenic, chlorinated dioxins, TCDD, furan, polychlorinated biphenyls, DDT and acrylamide are present in breast milk and infant formula foods (Mojska *et al.,* 2012; Lehmann *et al.,* 2018).In males, exposure to the dioxin TCDD between 1 to 9 years is associated with a reduction

in sperm counts and sperm motility (Mocarelli *et al.,* 2007) along with a decrease in Sertoli cell number in adulthood (Sharpe *et al.,* 2003).In female infants, exposure to polybrominated biphenyls via breast milk has been linked to early menarche (Blanck *et al.,* 2000). In addition to these more well-known EDCs, infants and children may be exposed to heat-induced food toxicants such as furan and acrylamide, which also have endocrine-disrupting potential (Robin and Clanci, 2007; FDA, 2004). Furan (C_4H_4O) belongs to a group of dioxins (polychlorinated dibenzo-furans, PCDFs). Furan is found in many treated food compounds, including jarred and canned foods, coffee and infant formulas (Pluim *et al.,* 1993; Karacaoğlu and Selmanoğlu, 2010b; Lehmann *et al.,* 2018).

The effect of developmental exposure to furan has been evaluated only in a handful of studies, despite strong experimental and epidemiological evidence that early life stages are highly sensitive to dioxins (Peterson *et al.,* 1993; Birnbaum and Tuomisto, 2000; Berga, 2008; van den Berg *et al.,* 2017). In addition to increased sensitivity, infants have higher intake rates of furan as compared to adults, because they rely solely on breastmilk or formula milk which contain high concentrations of furan. Exposure may even start in utero since furan readily passes the placental barrier (Van Wijnen *et al.,* 1990). A clinical study reported that 11-week old babies had higher thyroid hormone levels when their mother's breastmilk contained higher than average dioxin and furan concentrations (Pluim *et al.,* 1993). In adult rats, exposure to furan resulted in decreased testosterone levels, decreased sperm counts, impaired spermatogenesis and induced apoptosis in Leydig and germ cells (Karacaoğlu and Selmanoğlu, 2010b; Cooke *et al.,* 2014). The present study investigated the furan exposure effects at the neonatal stage. Investigated sexual maturation, sperm parameters, hormonal profile, histopathological parameters and brain monoamines levels.

MATERIALS AND METHODS

Animal care and maintenance

Animals were maintained according to the standard protocol as described above in chapter 1. At 10-11 weeks of age, estrous stage females (n=30) were kept in breeding cages containing a male rat. Vaginal smears were collected on consecutive days until the presence of sperm was confirmed. And was designated as day 1 of gestation (GD1). Pregnant females were kept in single cages until the birth of pups on gestational day 22 (GD22). The day of birth of the pups was considered as postnatal day 0 (PND0). The number of pups born was recorded, and the sex was determined by measuring the anogenital distance (AGD) using a stereomicroscope. Later pups were positioned in separate cages.

Experimental design

On PND0, male pups (n=50) were distributed into five treatment groups (10 pups per treatment group). The control group received a daily sc injection with corn oil (50 μL) from PND1-10, while treated groups received subcutaneous injections with furan (either 1, 5, 10 or 20 mg kg^{-1} d^{-1}) for ten days. Subcutaneous routes of furan administered were followed as neonatal rodents have low liver enzymatic activity, therefore there is no difference between oral and non-oral administration (Chapin *et al.*, 2008; Taylor *et al.*, 2008). Their developmental stage was monitored to study the following parameters.

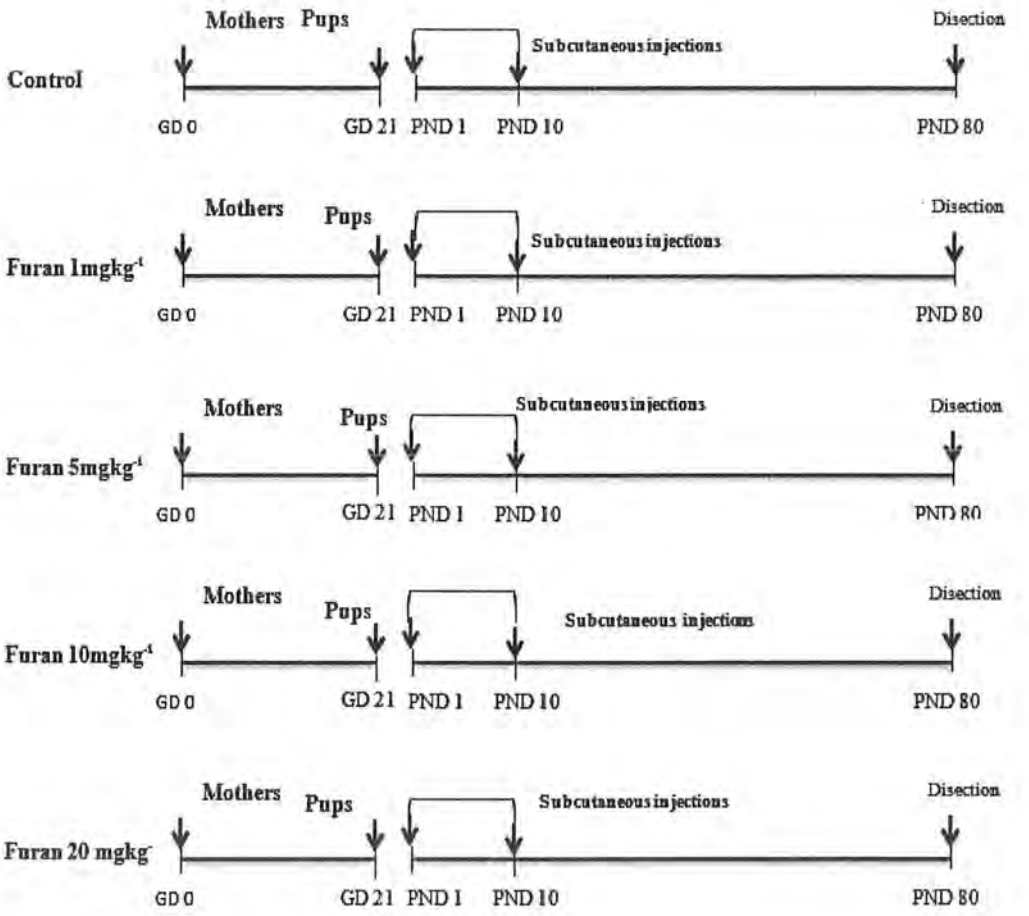


Fig 14. Schematic representation of neonatal exposure to furan.

Determination of body weight

Animals were weighed on PND30, PND45, PND80 and the day of preputial separation using weight balance.Net weight gain within each group was calculated.

Evaluation of sexual maturation

As a criterion for sexual maturation, the timing of preputial separation for male rats (beginning PND 35) was assessed and each pup was weighed when these criteria were achieved.

Evaluation of fertility

After the completion of the experiment. Take animals from each group and put in a separate cage with the untreated female rat. A vaginal smear from each paired female was collected in the four days following the pairing and the number of spermatozoa on it was counted. For the resulting litter, litter size, morbidity, and mortality were noted. Following formulas were used for calculating fertility.

Fertility (%) = {(number of pups in the male's litters) / (total number of pups) *100}

Animal euthanization

At PND 80, male rats (n=7 per group) were weighed, euthanized by using diethyl ether and collected the trunk blood. Testicular and epididymis organs were weighed and sort out for histological and sperm parameters.

Assessment of plasma biochemistry

As explained in chapter 2.

Sperm parameters

Sperm motility

As described in chapter 2.

Assessment of sperm viability

As described in chapter 2.

Epididymal sperm count (ESC)

As described in chapter 2.

Daily sperm production (DSP)

Take testicular tissues and removed the tunica albuginea. The tissue was homogenized in Triton X-100/(0.5 %) 5 mL NaCl (0.9 %) solution using a rotor for 30 seconds (Robb *et al.,* 1978). The homogenate was diluted 5 times, put the homogenate on the Neubauer chamber and counted the 19^{th} stage spermatids. The average of at least three readings was taken. To obtain the daily sperm production (DSP), the number of spermatids was divided by 6.3.

Hormonal analysis

Plasma T, LH, FSH, GH and cortisol were determined by ELISA kits; for LH, FSH and T (Reddot Biotech Inc. Canada), GH (Crystalchem, USA) and cortisol (Calbiotech, Inc. USA) according to the instructions in the kit.

Brain monoamines analysis

Brain monoamines were done according to the procedure as explained in chapter 2.

Tissue histopathology

As described in chapter 2.

Statistical analysis

The results were stated as mean ± SEM. lme4 (Bates *et al.*, 2014) and easyanova (Arnhold, 2013) package of R 3.2.5 (R Development Core Team, 2016) were used for statistics. The effect of the treatment was analyzed using one-way ANOVAs ('ea1' function of 'easyanova') with each of the measured physiological/histological parameters as a response variable and furan concentration as the dependent categorical variable. Post-hoc differences were computed with the Tukey Honest Significant Differences (the R function 'Tukey HSD). Correlation between plasma hormone concentrations of cortisol, plasma T, LH, FSH and GH were performed using Pearson's correlations. We checked each model's residuals for normality using the Shapiro-Wilk tests. Values of $P<0.05$ were considered statistically significant.

RESULTS

Effect of furan on body weight gain, accessory organ weight and abdominal fat pad.

All groups gained bodyweight from PND 1 to PND, but weight gain was significantly higher (Table 12, P=0.01). Weights of accessory reproductive organs (paired testis, paired epididymis, seminal vesicle, and prostate) were not significantly different among furan treatments (Table 12), although there was a tendency for increased weight. The weight of the abdominal fat pad was significantly(P=0.02) higher and the same tendency (although not statistically significant) in the 10 mg kg^{-1} group was observed (P=0.1, Table 12).

Effect of furan on plasma biochemical parameters, Onset of puberty and fertility % in adult rats.

Plasma concentrations of protein (P=0.06), triglycerides (P=0.001) and HDL (P =0.001) were significantly reduced in (20 mg kg^{-1}) (Table 13). Plasma cholesterol and LDL levels showed significant (P=0.06 and P =0.001, resp.) increases in the 5, 10 and 20 mg kg^{-1} dose groups (Table 13). The day of preputial separation was not significantly different among furan treatment groups (P=0.53; Table 13). We found a non-significant reduction in fertility in the high dose group (P=0.07, Table 13), and there was also a non-significant dose-dependent reduction in fertility % with increasing furan exposure (P=0.07, Table 13). No signs of morbidity and mortality were recorded throughout the experimental period.

Table 12. Effects of neonatal exposure to furan on body weight gain, accessory organ weight and abdominal fat pad weight of adult rats.

	Furan (mg kg^{-1})					
Parameters	**Control**	**1**	**5**	**10**	**20**	**Statistics**
Body weight gain (g)	236.16±6.01[a]	237.1±7.49[ab]	240.7±4.69[ab]	238.1±3.57[b]	243.7±8.87[b]	P=0.01 F=3.83
Paired testis weight (g)	1.73±0.05	1.70±0.14	1.69±0.09	1.71±0.08	1.68±0.06	P=0.9 F=0.04
Paired Epididymis weight (g)	0.94±0.12	0.80±0.09	0.76±0.06	0.71±0.10	0.65±0.04	P=0.60 F=0.69
Prostate (g)	0.47±0.06	0.46±0.17	0.44±0.19	0.47±0.15	0.44±0.08	P=0.99 F=0.01
Seminal Vesicle (g)	0.56±0.06	0.55±0.06	0.50±0.19	0.48±0.12	0.44±0.14	P=0.8 F=0.27
Abdominal fat pad (g)	1.24±0.02[b]	1.34±0.03[ab]	1.40±0.09[ab]	1.47±0.11[ab]	1.59±0.09[a]	P=0.03 F=2.96

P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different ($P<0.05$) in the rows compared to control with other treated groups.

[a]Values are presented as Mean ± (SEM)

[b]Significance at $P<0.05$ vs control

[c]Significance at $P<0.001$ vs control

Table 13. Effects of neonatal exposure to furan on biochemical parameters, puberty onset and fertility % of adult rats.

Parameters	Furan (mg kg^{-1})					
	Control	1	5	10	20	Statistics
Protein (mg 0.5 g^{-1})	5.51±0.20[a]	5.46±0.20[a]	5.47±0.06[a]	5.16±0.23[ab]	4.88±0.17[b]	P=0.03,F=2.95
Cholesterol (mg dL^{-1})	40±1.93	43.14±2.66	42±1.99	46.42±1.88	46±1.43	P=0.06,F=2.53
Triglyceride (mg dL^{-1}	53.28±1.29[a]	50.85±1.80[a]	44.57±0.37[b]	44.14±1.72[b]	44.42±1.65[b]	P=0.001,F=12.51
HDL (mg dl^{-1})	73±1.16[a]	72.14±1.26[a]	65.57±1.50[b]	65.71±1.99[b]	62.42±2.25[b]	P=0.001,F=12.47
LDL (mg dl^{-1})	29.54±1.66[b]	32.31±1.11[a]	39.54±2.15[ab]	34.94±2.09[b]	40.8±2.33[a]	P=0.001,F=7.75
Day of puberty onset	44.14±0.51	44.42±0.62	44±0.54	43.57±0.37	43.14±0.75	P=0.53,F=0.79
No of pups born/female	8±0.38	7.57±0.37	7.85±0.34	8±0.31	6.71±0.48	P=0.07,F=0.79
Fertility %	100±4.80	98.24±3.85	97.24±4.69	94.64±4.69	82.76±5.72	P=0.07,F=0.79

P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different ($P<0.05$) in the rows compared control with other treated groups.

[a]Values are presented as Mean (± SEM),

[b]Significance at $P<0.05$ vs control,

Effect of furan on sperm parameters

A dose-dependent decrease in DSP (P=0.06 and P=0.02) was observed (Table 14). Furan treatment also caused a reduction in sperm viability (P=0.003) and epididymal sperm count (P =0.05; Fig 15). A non-significant tendency for lower sperm motility was observed with increasing furan doses (P =0.16). Epididymal sperm count (caput, corpus, cauda) was significantly lower among the groups treated with the highest doses (P =0.09, P=0.004 and P =0.04 resp. in 5, 10, 20 mg kg^{-1} groups; Fig 15).

Table 14. Effects of neonatal exposure to furan on sperm parameters of adult rats.

	Furan (mg kg^{-1})					
	Control	**1**	**5**	**10**	**20**	**Statistics**
DSP ($\times 10^6$)	3.97±0.23[a]	3.75±0.77[a]	2.92±0.311[ab]	2.55±0.22[c]	2.37±0.07[c]	P=0.01 F=3.95
Sperm viability %	17.85±1.70[a]	14.28±1.17[ab]	13.14±1.37[ab]	12.71±1.58[ab]	9.28±0.94[b]	P=0.003 F=5.004
Sperm motility%	80.42±1.92	79.14±2.60	76.85±1.14	74.57±1.48	76.28±1.30	P=0.16 F=1.73

P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P<0.05) in the rows compared to control with other treated groups.

[a]Values are presented as Mean ± (SEM)

[b]Significance at P<0.05 vs control

[c]Significance at P<0.001 vs control

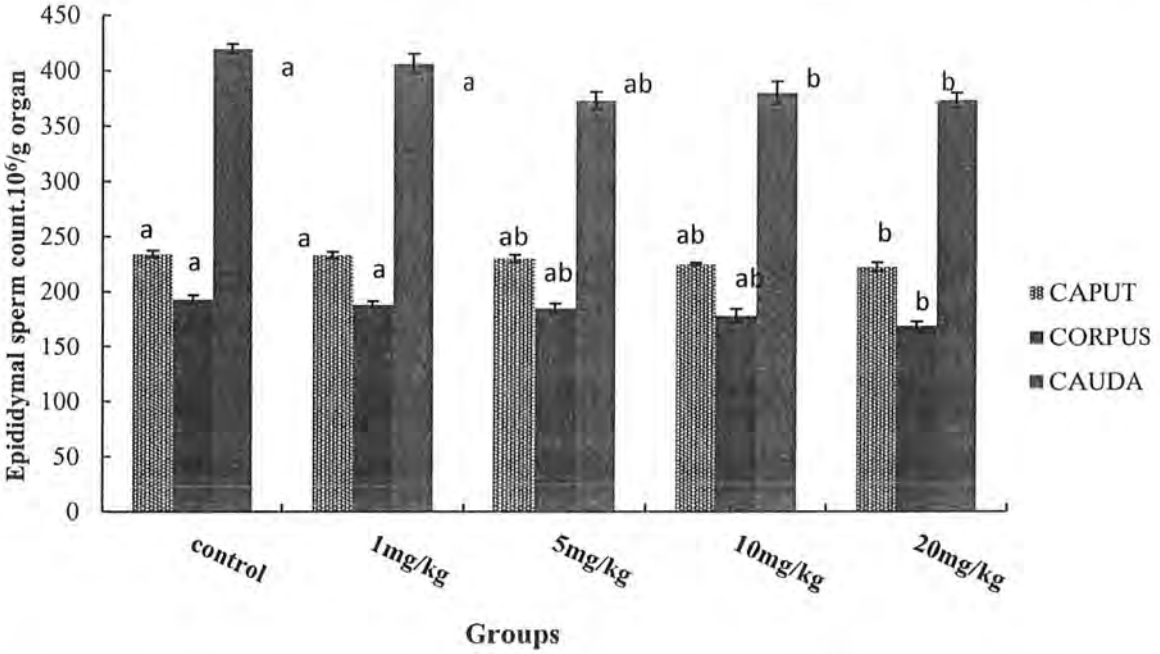


Fig. 15. Effects of neonatal exposure to furan on epididymal sperm counts (caput, corpus and cauda parts) in adult rats. Values represent mean ± SEM of (n=7). Letters above bars depict values that are significantly different ($P<0.05$) between different doses within the same epididymal part.

Effect of furan after neonatal exposure on hormonal profile

Plasma T concentration was reduced in the 10 (P=0.06) and 20 mg kg^{-1} d^{-1} (P=0.001) (Table 15). Similarly, significant reductions in plasma LH level (P=0.01) and GH concentrations (P=0.001) were observed in the groups receiving the two highest furan doses (Table 15). Cortisol concentrations were significantly elevated in the two high dose groups (P=0.001 and P=0.01 in the 10 and 20 mg kg^{-1} d^{-1} groups; Table 15).

To investigate the relationship between plasma cortisol concentration and plasma T, LH, FSH and GH concentrations, hormone concentrations were correlated in a pairwise fashion (Table 16, Fig 16). Results indicated that plasma cortisol concentration is negatively correlated with plasma testosterone (r=-0.385, P=0.006) and GH (r=-0.5131, P <0.01), while the relationship between cortisol and LH (r=-0.162, P =0.261) and cortisol and FSH (r=-0.155, P =0.281) was not significant. Moreover, T and LH, as well as T and GH correlated positively with each other (r= 0.379, P =0.007 and r = 0.560, P <0.001, resp.), While T and FSH were not correlated (r= 0.119, P=0.411). Finally, a positive correlation appeared between LH and FSH (r=0.298; P =0.03; Table 16; Fig 16).

Table 15. Effects of neonatal exposure to furan on hormonal profile of adult rats.

	Furan (mg kg^{-1})					
	Control	**1**	**5**	**10**	**20**	**Statistics**
Testosterone (ng ml^{-1})	4.45±0.20^{a}	4.28±0.025^{a}	4.13±0.17^{a}	3.83±0.16^{ab}	3.24±0.15^{b}	P=0.001 F=8.95
LH (ng ml^{-1})	1.79±0.04^{a}	1.74±0.04^{ab}	1.69±0.03^{ac}	1.63±0.03^{bc}	1.59±0.04^{c}	P=0.01 F=3.41
FSH (mlUml^{-1})	1.03±0.09	1.02±0.005	0.96±0.10	0.89±0.08	0.80±0.02	P=0.1 F=1.57
GH (ng ml^{-1})	210.7±1.75^{a}	203.6±2.13^{ab}	197.8±2.28^{bc}	190±3.62^{cd}	181.6±3.90^{d}	P=0.001 F=15.45
Cortiso (ng ml^{-1})	39.8±0.89^{c}	41.2±0.94^{bc}	44.1±0.88^{ac}	44.4±1.56^{ab}	46.5±1.05^{a}	P=0.001 F=5.94

P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different ($P<0.05$) in the rows compared to control with other treated groups.

[a]Values are presented as Mean ± (SEM)

[b]Significance at $P<0.05$ vs control

[c]Significance at $P<0.001$ vs control

Table 16. Pearson's correlations between plasma cortisol, T, LH, FSH and GH of adult rats.

	Correlation				
Parameters	**Cortisol (ng ml^{-1})**	**T(ng ml^{-1})**	**LH (ng ml^{-1})**	**FSH (mlUml^{-1})**	**GH (ng ml^{-1})**
Cortisol (ng ml^{-1})	r =1				
T(ng ml^{-1})	r = -0.385** P =0.006	r = 1			
LH (ng ml^{-1})	r = - 0.162 P =0.261	r = 0.379** P =0.007	r = 1		
FSH (mlUml^{-1})	r = -0.155 P =0.281	r = 0.119 P =0.411	r = 0.298* P =0.036	r = 1	
GH (ng ml^{-1})	r = -0.513** P =0.000	r = 0.560** P =0.00	r = 0.261 P =0.250	r = 0.102 P =0.482	r = 1

Pearson's correlation and sets significant relation; Pearson's correlation is shown with r whenever significant by P -value.

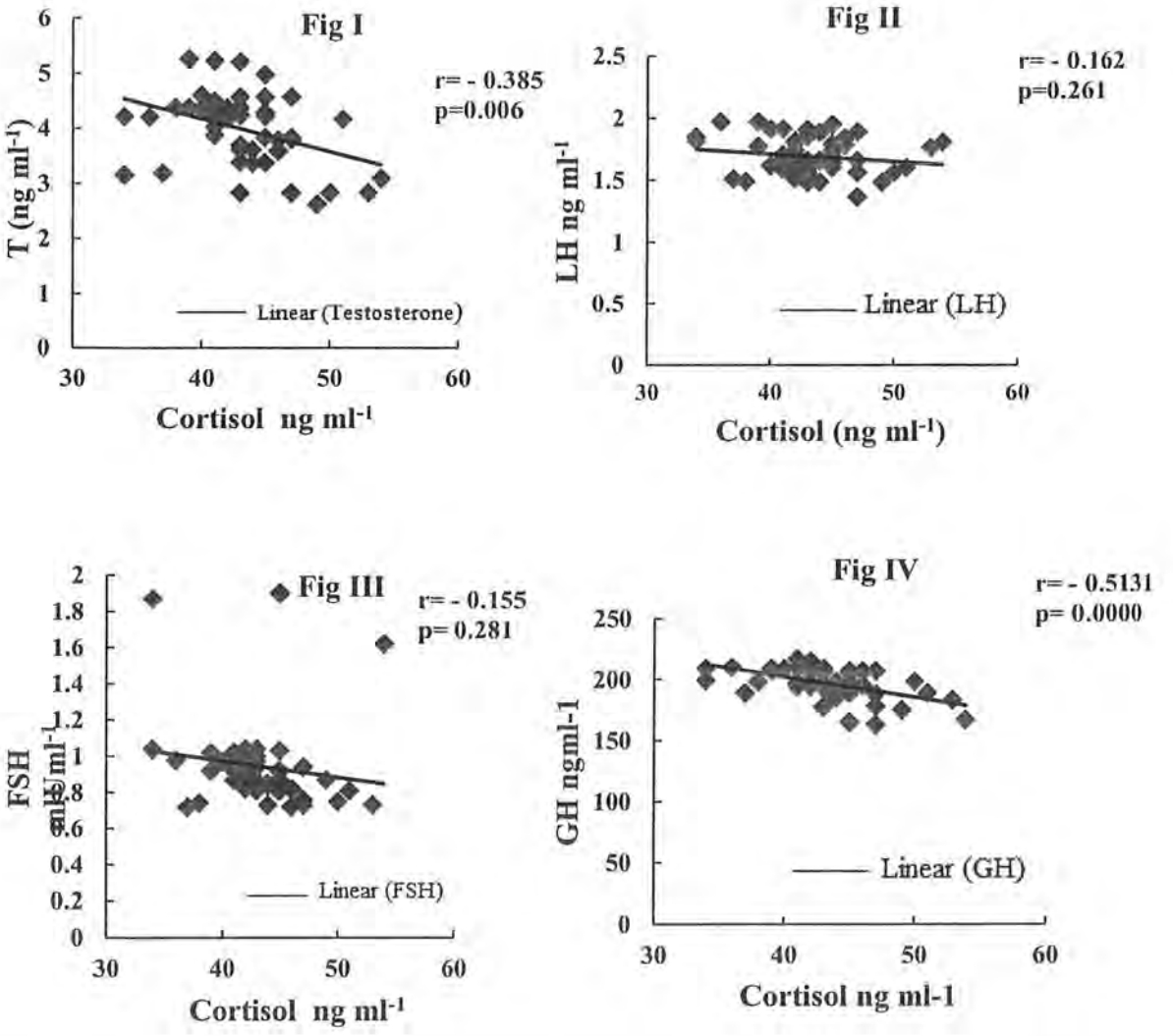


Fig 16. Plasma T (I), LH (II), FSH(II) and GH (IV) as a function of plasma cortisol concentration in adult rats exposed to furan. Pearson's correlation coefficient (r) and P values are provided in the top right of each graph.

Effect of furan after neonatal exposure on Brain monoamines levels

The effect of neonatal exposure to furan on brain hypothalamus was shown in Table 17. A significant ($P<0.001$) increase in 5HIAA/5HT levels were observed at high doses 10 and 20mgkg^{-1} groups of furan treatment in the brain hypothalamus region. Similarly, some alterations in the dopaminergic (DOPAC / DA and HVA/ DA) levels were observed but these alterations were not statistically significant. After furan exposure, a significant ($P=0.04$) increase was observed in NE level. All brain monoamines showed a positive correlation with the cortisol level (Table 17). Results indicated that plasma cortisol concentration is positively correlated with brain serotonergic activity (5HIAA/5HT ratio) ($r=0.525$, $P= 0.001$**) and dopaminergic activity (DOPAC/DA ratio) ($r=-0.34$, $P=0.015$), (HVA/DA ratio) ($r =0.35$, $P=0.012$), and NE ($r =0.447$, $P=0.004$) level. While the relationship between all brain monoamines are positively correlated with each other. Finally, a positive correlation appeared between cortisol and brain monoamines. (Table 18, Fig 17).

Table 17. Effects of neonatal exposure to furan on the concentration of monoamine in hypothalamus tissue of adult rats.

	Furan mg kg^{-1}					
	Control	**1**	**5**	**10**	**20**	**Statistics**
5HIAA /5HT ratio	0.501±0.02[b]	0.504±0.02[b]	0.514±0.04[b]	0.611±0.03[a]	0.656±0.03[a]	P=0.002 F=4.89
DOPAC / DA ratio	0.23±0.05	0.26±0.03	0.27±0.01	0.28±0.03	0.36±0.05	P=0.25 F=1.37
HVA / DA ratio	0.372±0.01	0.374±0.03	0.38±0.02	0.39±0.04	0.49±0.07	P=0.28 F=1.29
NE (ng g^{-1})	249±13.21[a]	227±16.23[a]	214±22.73[a]	190±8.96[a]	190±13.48[a]	P=0.04 F=2.61

P and F values in the rows were obtained from ANOVA with completely randomized designs, followed by Tukey's post hoc analysis, which shows a pairwise comparison of control and furan-treated groups. Mean values with different superscripts show a significant difference ($P<0.05$) in the rows when comparing control with other treatment groups.

Table 18. Correlations of serum cortisol and brain monoamine concentrations in rats.

	Correlations				
	Cortisol (ng ml^{-1})	**5HIAA/5HT ratio**	**DOPAC/DA ratio**	**HVA/DA ratio**	**NE**
Cortisol (ng ml^{-1})	r =1				
5HIAA/5HT ratio	r=0.525** P= 0.001	r =1			
DOPAC/DA ratio	r = 0.342* P=0.015	r =0.201 P=0.163	r =1		
HVA/DA ratio	r =0.356* P=0.012	r=0.142 P=0.324	r=0.105 P=0.470	r =1	
NE (ng g^{-1})	r = 0.447** P=0.004	r=0.182 P= 0.206	r=0.119 P=0.411	r= 0.163 P=0.258	r =1

Pearson's correlation and sets significant relation; Pearson's correlation is shown with r whenever significant by p-value.

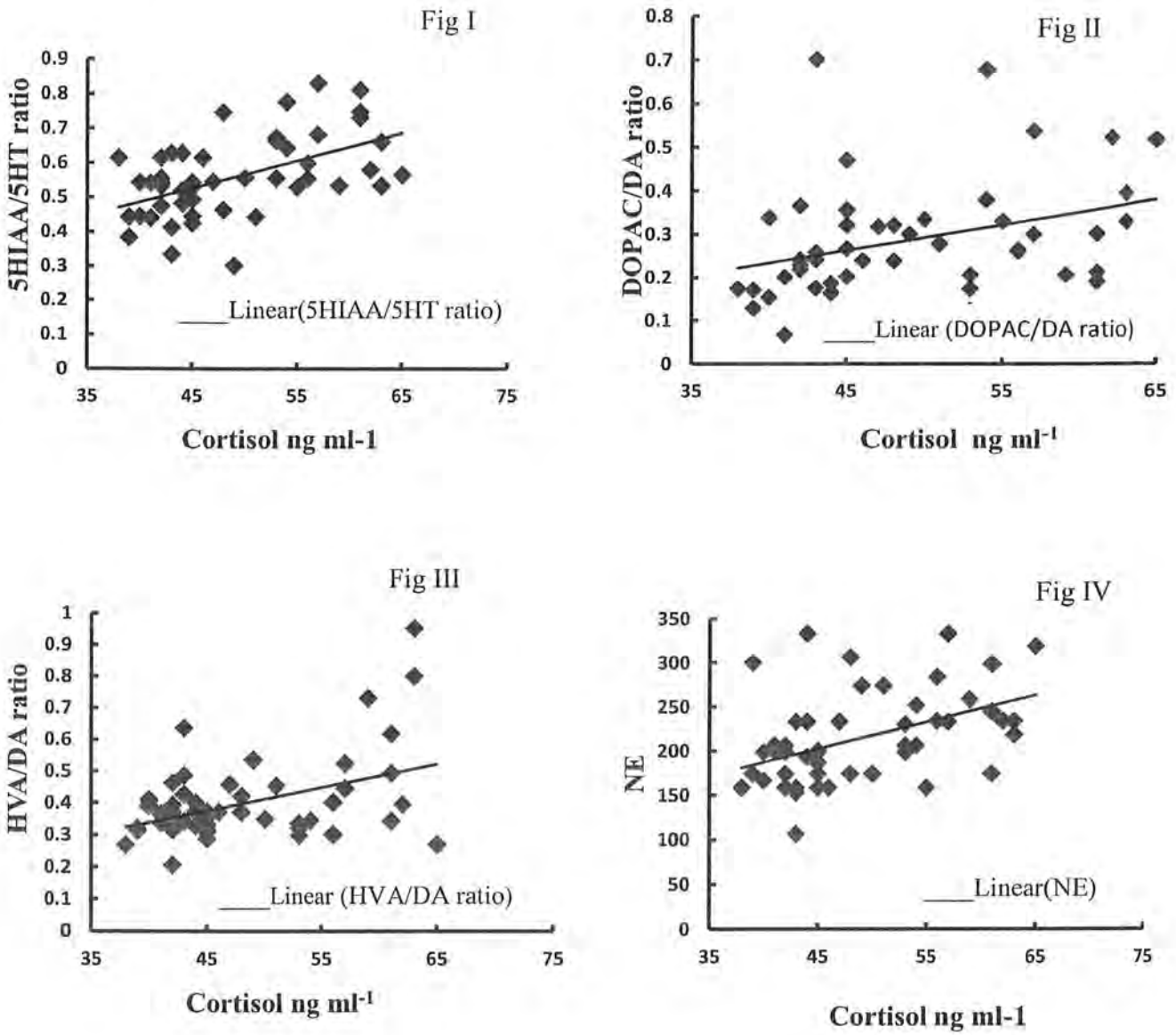


Fig 17. Brain monoaminergic activities 5HIAA /5HT ratio (I) DOPAC / DA ratio (II) HVA / DA ratio and NE (IV) as a function of plasma cortisol concentration in rats neonatally exposed to furan. Pearson's correlation coefficient (r) and P values are provided in the top right of each graph.

Effect of furan on testicular histopathology

Analysis of testicular histology indicated a significant reduction in the percentage area covered by seminiferous tubules in the two high treatment groups (Table 19; P =0.01 and P = 0.002 for 10 and 20 mg kg^{-1}, resp.) While the area covered by interstitial spaces increased significantly. The changes were in the same direction for the other variables measured but were not statistically significant here (Fig 18, Table 19).

Table 19. Effects of neonatal exposure to furan on testicular tissue histopathology of adult rats.

Parameters	Furan (mg kg^{-1})					
	Control	1	5	10	20	Statistics
Area of seminiferous tubule %	89.18±0.63[a]	87.05±0.78[ab]	86.11±0.90[ab]	84.97±1.26[b]	84.35±0.94[b]	P=0.002 F=4.25
Area of interstitium %	12.66±0.58[a]	13.89±0.52[a]	14.56±0.49[b]	17.20±0.59[bc]	17.84±0.56[c]	P=0.001 F=16.18
Seminiferous tubule diameter (µm)	234.26±6.82	227.43±6.73	223 ±10.49	223.42±7.38	218.88±9.65	P=0.7 F=0.49
Epithelial height (µm)	63.23±2.51	60.04±2.87	61.84±2.88	58.11±2.42	58.51±2.56	P=0.60 F=0.68

P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P <0.05) in the rows compared to control with other treated groups.

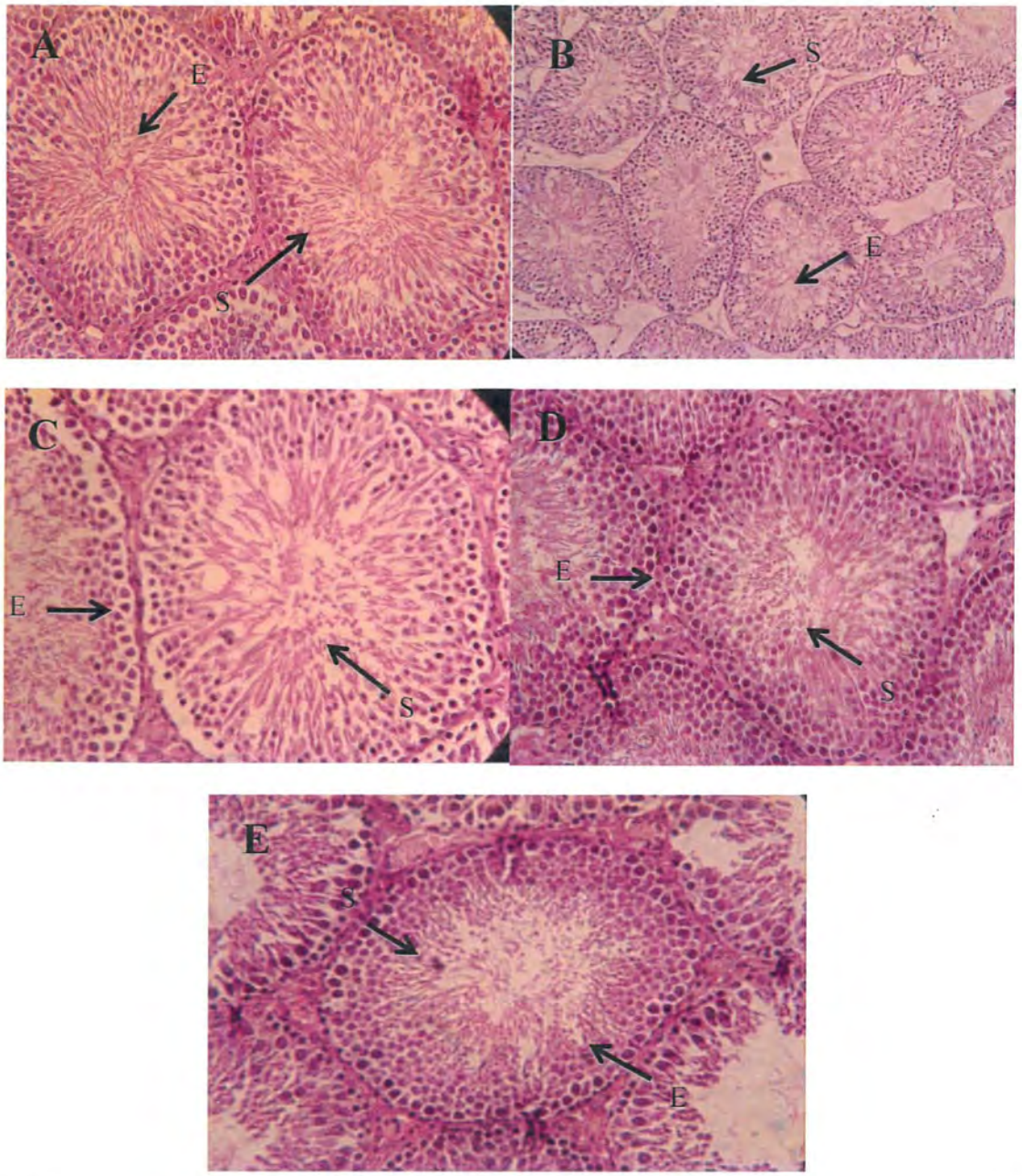


Fig. 18. Photomicrograph (40× magnification) of seminiferous tubules (hematoxylin-eosin stain) of male rats exposed to different treatments of furan at the neonatal stage. (A) control showing regular normal germ cells; (B) 1 mg kg^{-1} showing normal spermatogenesis; (C) 5 mg kg^{-1} displays normal morphology with a slightly widened lumen; (D) 10 mg kg^{-1} showing increased lumen diameter and normal epithelial height; (E) 20 mg kg^{-1} showing a thin degenerated epithelium with the empty lumen. Letters indicate spermatozoa (S), and epithelium (E).

Effect of furan on cells in seminiferous tubules

Spermatogonia present in the seminiferous tubules was significantly reduced (Table 20; P =0.003, P =0.001 & P =0.001 in 5, 10, 20 mg kg^{-1} doses, resp.). Similarly, a significant (P=0.001) reduction in several spermatocytes was also observed in the two highest dose groups (10 mg kg^{-1} and 20 mg kg^{-1}). Besides, spermatids were reduced in the 20 mg kg^{-1} dose group (P =0.04; Table 20).

Table 20. Effects of neonatal exposure to furan on different types of cells in seminiferous tubules of testes of adult rats.

	Furan (mg kg^{-1})					
Cells number (n)	**Control**	**1**	**5**	**10**	**20**	**Statistics**
Spermatogonia	61.4±1.023[a]	58.6±0.75[ab]	56.6±0.80b[c]	55.64±0.98[bc]	53.08±1.06[c]	P=0.001 F=11.21
Spermatocytes	81.08±1.165[a]	78.2±1.50[ab]	77.48±1.137[ab]	77.12±0.83[at]	75.58±1.06[b]	P=0.01 F=3.03
Spermatids	257.52±2.14	255.84±2.24	253.56±2.34	253.32±1.92	248.8±2.39	P=0.06 F=2.20

Values are presented as Mean ± SEM (n=7). P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc show a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P <0.05) in the rows compared to control with other treated groups.

Effect of furan on epididymis histopathology

No significant morphometric alterations were detected in the histology of epididymis caput tissue of adult rats. There was, however a trend for a reduction in tubular and lumen diameters and an increase in epithelial height in the high dose group (Table 21; Fig 19). A similar pattern was observed in the cauda epididymis (Table 22; Fig 20) although here, epithelial height was significantly increased (P=0.05). No significant difference was recorded in other parameters of the epididymis in treated groups when compared to control. No differences between treatment groups were observed for epididymis corpus histology.

Table 21. Effect of neonatal exposure to furan on caput epididymis tissue histopathology of adult rats.

	Furan (mg kg^{-1})					
Parameters	**Control**	**1**	**5**	**10**	**20**	**Statistics**
Tubule diameter (µm)	354.4±8.33	350.08±7.26	347.28±8.12	339.76±9.83	343.9±10.04	P=0.80 F=0.40
Lumen diameter (µm)	255.28±7.83	252.14±7.27	253.36±8.05	246.6±9.31	237.84±6.32	P=0.51 F=0.81
Epithelial heights (µm)	31.40±0.96	29.00±0.90	29.47±1.02	28.20±0.76	29.16±0.82	P=0.14 F=1.73
Epithelium % age	39.25±1.20	36.25±1.13	36.84±1.28	35.25±0.95	36.46±1.03	P=0.143 F=1.73
Lumen% age	60.74±1.20^{b}	63.74±1.13^{ab}	63.15±1.28^{a}	64.74±0.95^{ab}	63.53±1.03^{a} ^{b}	P=0.03 F=2.62

Values are presented as Mean ± SEM (n=7). P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc show a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P<0.05) in the rows compared to control with other treated groups.

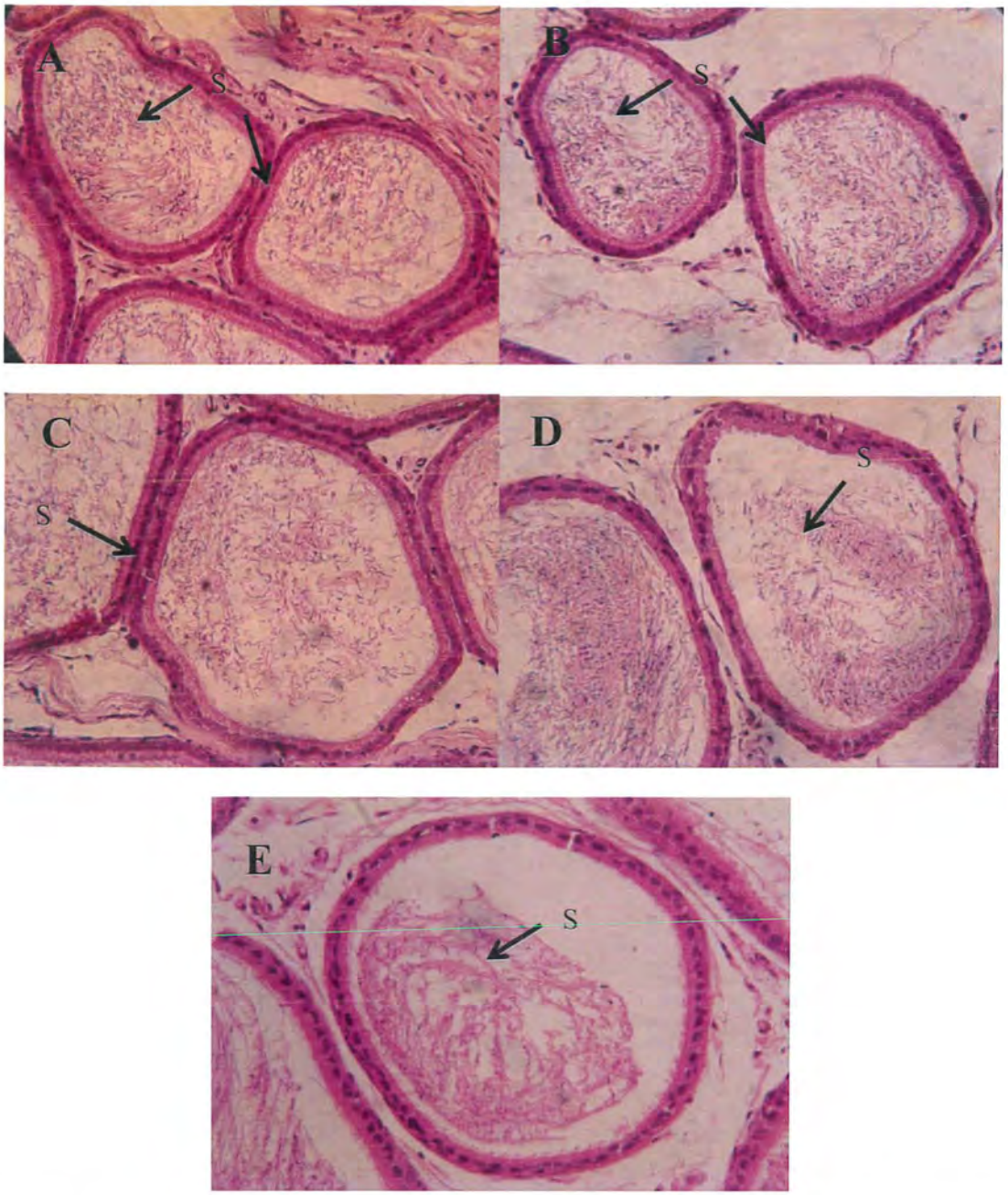


Fig 19. Photomicrograph (40X magnification) of caput epididymis region (hematoxylin-eosin stain) male rats exposed to treatments of furan at neonatal stage. (H&E, 40X) from: (A) Control group; with normal morphology (B) 1 mg kg^{-1} group; with thin epithelium-lined with stereocilia, (C) 5 mg kg^{-1} group; showing normal spermatozoa in lumen (D) 10 mg kg^{-1} group; showing decrease in concentration of spermatozoa, (E) 20 mg kg^{-1} group; Slight reduction in spermatozoa. Letters indicate spermatozoa (S), and epithelium (E).

Table 22. Effects of neonatal exposure to furan on caudal epididymis tissue histopathology of adult rats.

Parameters	Furan (mg kg^{-1})					
	Control	1	5	10	20	Statistics
Tubule diameter (µm)	454.48±7.13	453.22±8.91	449.24±9.30	449.42±10.09	445.7±10.54	P=0.9
						F=0.14
Lumen diameter (µm)	415.2 ±10.99	417.7 ± 8.24	413.3 ± 7.83	409.14±9.57	404.98±11.96	P=0.90
						F=0.26
Epithelial heights (µm)	27±1.29^{a}	25.03±1.27^{ab}	25.96±1.40^{ab}	23.14±1.69^{ab}	22.03±1.29^{b}	P=0.05
						F=2.31
Epithelium % age	34.14±1.61^{a}	31.29±1.58^{ab}	32.45±1.75^{ab}	28.93±2.12^{ab}	27.54±1.61^{b}	P=0.05
						F=2.35
Lumen% age	65.85±1.61^{b}	68.70±1.58^{ab}	67.54±1.75^{ab}	71.06±2.12^{ab}	72.45±1.61^{a}	P=0.05
						F=2.30

P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P<0.05) in the rows compared to control with other treated groups.

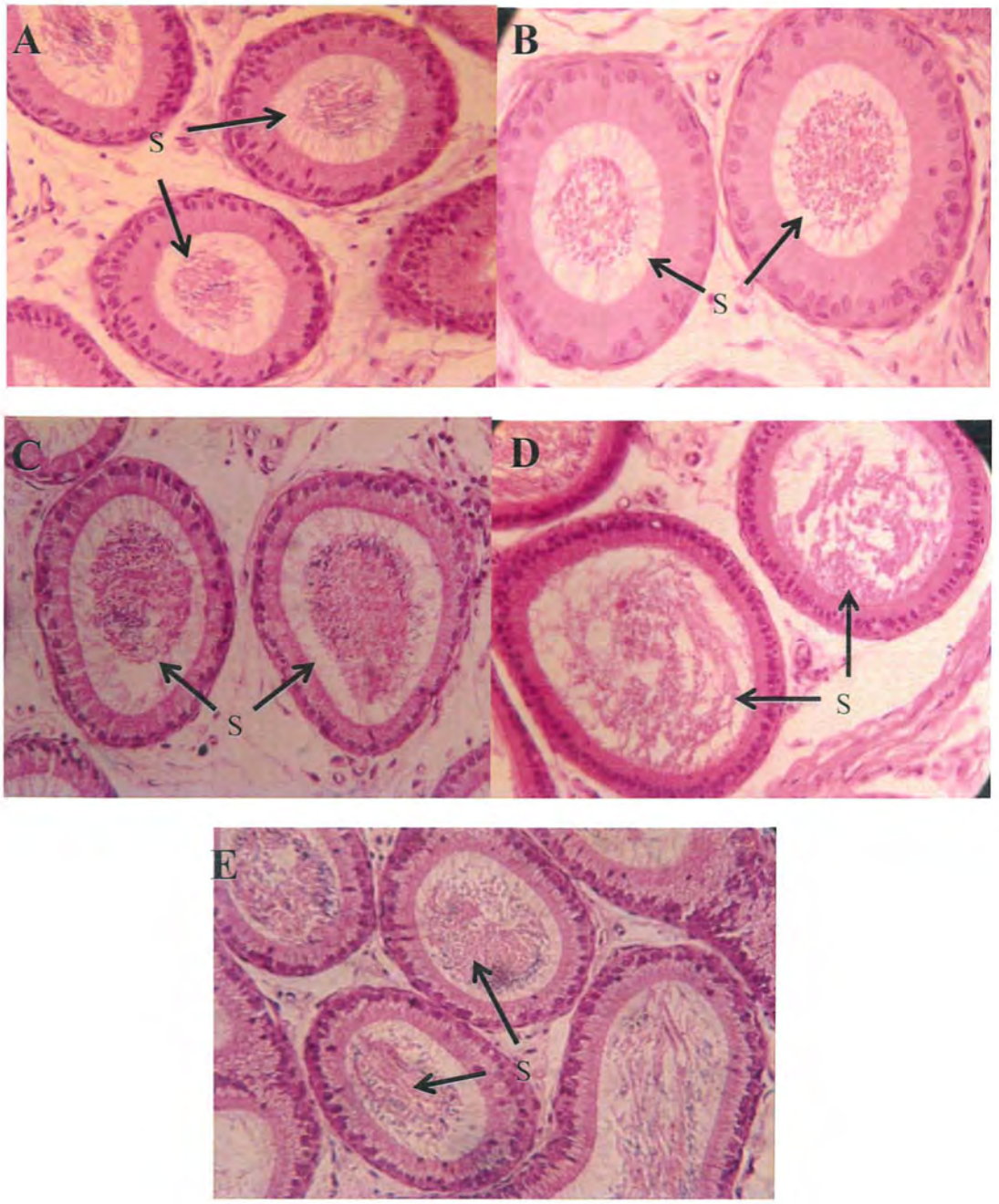


Fig 20. Photomicrograph (40× magnification) of cauda epididymis region (hematoxylin-eosin stain) of male rats exposed to different doses of furan at the neonatal stage. (A) Control; demonstrating normal morphology of cauda epididymis; lumen filled with mature sperms, (B) 1 mg kg^{-1} group; showing normal morphology like control. (C) 5 mg kg^{-1} group; showing slight changes in the morphology of tubules, surrounded by stroma. (D) 10 mg kg^{-1} group; lumen has a little number of spermatozoa. (E) 20 mg kg^{-1} group; showing a further increase in epithelial height and little lumen sperm concentration. Letters indicate spermatozoa (S)and epithelium (E).

DISCUSSION

Few animal studies have investigated the possible endocrine-disrupting effects of furan. The current study is the initial experiment that exposed rats to furan at the neonatal stage of life and estimated the effects on the male reproductive system in adulthood. Neonatal male rats were subcutaneously injected with four different furan doses, to investigate the effects in adult male rats (PND 80). We find clear evidence that exposure to the maximum doses of furan broadly and consistently affects the development of the male reproductive system, ultimately compromising male fertility in adulthood. The most prominent effects we observed were dose-dependent increases in body weight, decreased plasma T, LH and GH, increased cortisol levels, and decreased sperm counts associated with altered testicular and epididymal histology.

Elevated weight gain has been observed in an earlier study where rats were exposed to furan from the weaning period to post-puberty (Karacaoğlu and Selmanoğlu, 2010a) and in suckling mice after furan treatment (Blagburn *et al.*, 1998). The mechanism of how furan affects body weight gain is unknown, but one can speculate that it could be the result of a direct effect on nutrition uptake or an indirect effect via the observed changes in plasma hormones. (Mårin *et al.*, 1992) has suggested that cortisol can cause obesity, an idea that has been further developed by later studies (FTC, 2004; Abraham *et al.*, 2013). Also, low testosterone levels are associated with adiposity resulting from to metabolic impairments (Fui *et al.*, 2014), while testosterone replacement therapy instigated a reduction in fat mass and prominent increase in fat-free mass in both hypogonadal and normal men (Gulve and Spina, 1995; Cox *et al.*, 1999; Evans, 2000). Rasmussen (2010) reported that GH deficiency is related to increased fat mass and abdominal fat mass accumulation (Rasmussen, 2010). Hence, the increase in weight gain and abdominal fat mass found in the present study in those groups receiving the highest dose (20 mg kg^{-1}) may well have been caused by the elevation of cortisol levels and the decreased level of testosterone, LH and GH.

The relationships observed between plasma hormone levels furthermore fit well with earlier studies reporting negative relationships between plasma cortisol vs. testosterone or GH (Wennink *et al.*, 1990). Indeed, many components of the gonadal axis are downregulated by plasma glucocorticoids, either by affecting hypothalamus and

pituitary functions or by affecting the responsiveness of target tissues to gonadal hormones (Thakore and Dinan, 1994; Borges *et al.,* 1997). Increased cortisol secretion has been associated with a decreased production of sex steroids and GH in a multitude of studies (Burguera *et al.,* 1990; Björntorp, 1995; Chen *et al.,* 1997; Tsigos and Chrousos, 2002; Viau, 2002; Liening and Josephs, 2010). High doses of glucocorticoids inhibited testicular Leydig cell function in rats (BAMBINO and HSUEH, 1981).In humans, exposure to cortisol caused a marked decrease in testosterone production (Cumming *et al.,* 1983). A decrease in the testosterone production in adulthood is a recurrent finding in studies exposing rats to heat-induced food toxicants (Salian *et al.,* 2009; Karacaoğlu and Selmanoğlu, 2010b; El-Akabawy and El-Sherif, 2016). Also, endocrine disruptors such as PAHs and PCBs, as well as lead and cadmium cause elevated cortisol levels and decreased GH levels in fish and mammals (Tort *et al.,* 1996; Tan *et al.,* 2007; Zimmer *et al.,* 2009; Sajjad *et al.,* 2018). This suggests that the growth of the reproductive organs can be disturbed by reducing testosterone, which in turn could be driven by increased cortisol levels.

EDCs and Environmental toxicant have been the neurotoxic effects and can interrupt the of neurotransmitters release, synthesis and transport (Dickerson and Gore, 2007; Rasier *et al.,* 2007). EDCs and non-EDCs might display neurotoxic effects through different pathways (Schug *et al.,* 2015). Ali, et al (1983) suggested that acrylamide toxicant resulted in superior amounts of 5-hydroxyindolacetic acid in all parts of the brain. Numerous studies specify that Pb and Cd exposure during the development of the CNS alter the actions of the monoaminergic structure (Bressler and Goldstein, 1991; Antonio *et al.,* 1996; Mejia *et al.,* 1997; Antonio *et al.,* 1998; Antonio *et al.,* 1999; Antonio *et al.,* 2002). Pb and Cd co-exposure during perinatal mainly produced variations in hippocampus serotoninergic and dopaminergic activity (Leret *et al.,* 2003).

Stress indicator is cortisol production. In many earlier studies, it was also reported that environmental chemicals caused neurotoxicity through proper mechanisms increased production of cortisol and ROS level(Chattopadhyay *et al.,* 2002a; Chattopadhyay *et al.,* 2002b; García-Chávez *et al.,* 2003; Rao and Avani, 2004; Wang *et al.,* 2018). In fact, hyperactivity of the HPA axis may damage monoaminergic neurons (Wang *et al.,* 2018).

In several studies, suggested that brain 5-HT is involved in HPA and HPI axes regulation (Dinan, 1996; Winberg *et al.,* 1997; Winberg and Lepage, 1998; Hoglund *et al.,* 2000; Heisler *et al.,* 2007). Further, the activity of the HPA axis and 5HT are involved with each other's (Curtis and Patel, 2008). It has been previously reported that neuroendocrinology of reproduction control by neurotransmitters (Gallo, 1980; Nock and Feder, 1981). In a current study the brain monoamines that might be regulated by the neurotransmitters. Reproductive functions in mammals are closely associated with serotonin neurotransmitter (Prasad *et al.,* 2015). 5HT neurons enhance gonadotropin release (Spinelli, 2000). The LH level in the current study in high doses of furan that might be the regulation of the elevated level of serotonin, Similar finding were reported by (Rubinow and Schmidt, 2006).

We found a consistent decrease in both the quality and number of sperm from males neonatally exposed to the two highest doses of furan. A reduction in sperm activities was observed in the high treatment group, daily sperm production and epididymis (caput, corpus and cauda) sperm counts were also reduced in a dose-dependent manner. These findings may be explained by the lower testosterone and LH concentrations. The elevated cortisol levels may have contributed to the inhibition of spermatogenesis, disturbance of spermiation and impairment of sperm quality (Castranova *et al.,* 2005; Pressman *et al.,* 2018). Also, furan exposure at the weaning stage of life resulted in germ cells and Leydig cells apoptosis and spermatogenic weakening, which was associated with lower LH and testosterone levels (Karacaoğlu and Selmanoğlu, 2010b).

Histological analysis of the showed alterations in testicular morphology due to furan effects. In the groups receiving the highest doses, prominent multilayered spermatogonia were recorded, while seminiferous tubules lumen was not heavily packed with elongated spermatozoa. These findings are following earlier reports of furan exposure (Karacaoğlu and Selmanoğlu, 2010b). In contrast to the testes, morphometric findings of the epididymis also showed few amendments.

We observed increases in plasma cholesterol and LDL while triglyceride (TG) and HDL were decreased, findings that are in line with the observed increases in body mass and abdominal fat. The increased production of fatty acids leads to a reduction in

plasma HDL and elevation in plasma cholesterol concentrations, which can ultimately result in liver dysfunction. Lipoproteins (HDL and LDL) are therefore considered sensitive biomarkers of liver function (Rawi *et al.,* 2012).TG/HDL ratio is clinically used for the detection of apparently healthy individuals with cardiovascular or metabolic impairments (Murguía-Romero *et al.,* 2013). The lowest levels of HDL are detected in patients with fasting chylomicronæmia and hypertriglyceridemic subjects, suggesting an inverse relationship between the metabolism of triglyceride and HDL (Schaefer *et al.,* 1978). However, the present study detected a decrease in both triglyceride and HDL associated with furan treatment, which could be explained by the observation that plasma lipoprotein levels often remain low during the early stages of development (Robinson and Seakins, 1963). An alternative explanation for the observed changes in plasma lipoproteins stems from the observation that several liver toxins have been reported to inhibit the release of hepatic triglyceride into the bloodstream. Acrylamide, for example, also causes changes in lipoproteins in the same direction (Rawi *et al.,* 2012). This would explain the observed low plasma triglyceride concentration and could also have led to a the buildup of triglyceride in the liver (Robinson, 1973), which has been associated with liver damage and fibrosis in obese mice (Yamaguchi *et al.,* 2007). In the latter study male rats were exposed to furan, which also resulted in elevated plasma LDL levels. Cholesterol enters the normal body cells by the permission of LDL receptors. Formation of new LDL receptors was eliminated because of cholesterol (Elaine, 2009). Excess levels of free cholesterol inhibit cholesterol and LDL receptor synthesis, thus reducing LDL uptake which promotes cholesterol storage. Restricting the LDL uptake and non-functioning of its receptors enhance the serum cholesterol levels (Linda, 2012).

Also, the reduction in plasma total protein levels can be regarded as evidence for a reduction in liver function, as previously reported by (Karakilcik *et al.,* 2004; Alam *et al.,* 2017). About 6% to 7% of the blood plasma proteins, including albumin, fibrinogen and prothrombin are synthesized in the liver (Osserman and Takatsuki, 1963). According to previous studies, hypoalbuminemia is associated with advanced chronic liver diseases (Koneri *et al.,* 2008). Liver damage could occur via peroxidation of polyunsaturated fatty acids, which are detrimental to cellular homeostasis, through the formation of aldehydes. Aldehydes impair nucleotide and protein synthesis, increase the production of the TNF-α, and promote an influx of

inflammatory cells into the liver, leading to collagen deposition, hepatic injury and inflammatory response (Esterbauer *et al.*, 1991; Yamauchi *et al.*, 2003).

Importantly, we observed a dose-dependent decline in fertility (number of pups conceived) in male rats neonatally exposed to furan. This finding can be adequately explained by the lower testosterone levels that arguably resulted in lower sperm numbers and quality, which were produced by testes showing clear histological abnormalities. We find no evidence that furan exposure caused a delay in the onset of puberty (as determined from preputial separation), which has been observed for ethynylestradiol, among other EDCs (Yoshimura *et al.*, 2005). Results are the same as prior studies in which genistein exposure showed no effects on the onset of puberty, but the decline in the number of pups produced (Nagao *et al.*, 2001).

CONCLUSION

The present study revealed that exposure to the highest doses of furan broadly and consistently affects the development of the male reproductive system, which ultimately lead to a reduction in fertility. Our results are in line with previous studies on furan exposure at the weaning stage and highlight that developmental exposure to furan can cause organizational effects on the development of male reproductive systems. This study provides important preclinical data on the minimal furan dose at which endocrine disruption can occur. Our results also warrant investigation of the reproductive system of female rats, as well as a screening for other endocrine effects unrelated to reproductive systems.

Chapter No. 4

Effects of endocrine disruptor furan on reproductive physiology of rats - An F1, extended one generation reproductive toxicity (EOGRTs) study.

ABSTRACT

The present study investigated the reproductive toxicity of furan in EOGRTS. Sprague Dawley F0 weaning rats (30/sex/group) were exposed to furan orally at 0, 1, 2.5, 5 and 10 mg kg^{-1} for ten weeks (males), two weeks (females) and then mated. Results of F0 indicated that in the furan (5 and 10 mg kg^{-1}) treated groups body weight gain, decreased during pre-breed & gestational period while increased during lactation periods. F0 animals pre-breeding exposure resulted in foot splay and head tilt at (10 mg kg^{-1}). At the birth number of live pups were decreased. At PND 70 a decrease (P=0.03) among male rats was seen in testosterone (T) level in 10 mg kg^{-1} furan treated group in the F1 generation. FSH and LH levels were also reduced. The testicular weight was reduced in the F1 generation at PND 70 with decreased (P=0.01) daily sperm production (DSP) in the higher dose furan group. Some histopathological changes were also observed in the testis in groups whose parents were previously exposed to 10 mg kg^{-1}bw of furan. Findings suggested that exposure to food-based contaminant furan induced remarkable changes in the F0 (Parental stage) and F1 (Offspring, pubertal & adult stage) generation of rats.

INTRODUCTION

Furan (C4H4O) is a colourless volatile organic compound that is in practice in several industries and also formed in various common foods products that experience excessive temperature dealing e.g canned and coffee, sauces, soups, infant formulae and beverages (FDA, 2004b) (Conti *et al.*, 2014) (Bakhiya and Appel, 2010; Moro *et al.*, 2012; Cooke *et al.*, 2014). Furan is also present in the environment as the main constituent of cigarette smoke, wood smoke, and exhaust gases from engines (Rehman *et al.*, 2019a). Because of the toxic nature of furan and its excessive daily intake in humans through diet and air, it has become a matter of concern. The FDA has reported in various surveys the presence of furan in eatable stuff (FDA, 2004a, 2005). The European Food Safety Authority also reported that among adults, prepared coffee is the main source of furan (EFSA, 2010). IARC and NTP have categorized furan a carcinogenic compound for health IARC, 1995:NTP,1993). Previous observations reported that furan caused various types of cancer and has adverse effect on the biological system of humans and animals (IARC, 1995; Hamadeh *et al.*, 2004; Pandir, 2015b; Uçar and Pandir, 2017).

Furan needs to be removed due to its adverse effects on health (Rehman *et al.*, 2019a). Various toxicants are dangerous to cell membranes since they cause an increase of lipid peroxidation (LPO), production an increasing oxygen species (ROS) and, NA damage in the cells (Farokhi *et al.*, 2012; Cooke *et al.*, 2014; Uçar and Pandir, 2017). Furan can easily pass through biological membranes due to its low polarity and acts as an endocrine disruptor as it has been reported previously that experience to different concentrations of furan resulted in the disturbance of the reproductive neuroendocrine system in rats (Cooke *et al.*, 2014). It has been earlier stated that furan is cytotoxic, genotoxic, and an apoptotic inducer (Heppner and Schlatter, 2007; Karacaoğlu and Selmanoğlu, 2010). Previous literature on furan reported that its exposure causes reproductive impairment by disturbing reproductive organ growth, spermatogenesis, T, LH and FSH secretion and reproductive performances (Karacaoğlu and Selmanoğlu, 2010; Cooke *et al.*, 2014; Rehman *et al.*, 2019a). Previously reported that hazardous environmental toxicants can bring variations in the cell's signalling pathways and defensive and protective systems of the antioxidant levels of the cells by affecting physiological conditions (Pandir, 2015a; Baş *et al.*, 2016). Furan caused significant

changes in the histological structure of cells, malondialdehyde levels, antioxidant enzyme activities and triggered DNA by producing reactive oxygen species (ROS) (Pandir, 2015b; Uçar and Pandir, 2017; Rehman *et al.*, 2019a). Furan prominent effects were reported in the reproductive systems of males and females (El-Akabawy and El-Sherif, 2016; Kara *et al.*, 2016; Uçar and Pandir, 2017; Rehman *et al.*, 2019b). However, a study was conducted on Taiwanese youngsters, their mothers used Polychlorinated biphenyls and furan rich contaminated rice oil resulted in, minor size of the penis in comparison with age-matched control boys (Guo *et al.*, 1995). Similarly, Guo *et al.* (2000) reported that prenatally exposed young men with PCBs and furan have abnormal sperms (Guo *et al.*, 2000). Tyla *et al.* (2000) previously observed the reproductive toxicity of food toxicant acrylamide in the next two generations of fisher (F344) rats. Furan was also reported to occur in morning urine samples of humans in different concentrations (Ghosh *et al.*, 2015).

Previously, many studies revealed the reproductive toxicity of furan in adults' rats. Thus far, no information is available in the literature about the toxic effects of furan in the generation. The current study was designed to study the (EOGRTS) across different life stages (Parental, Offspring and Adults). The main reason of this EOGRTS was to assess the possible effects of furan on F0 (parental) reproduction, and F1(First generation) offspring growth and development with calculations of endocrine function following exposure during the early period of development. This EOGRTS design was executed in compliance with Environmental Protection Agency (OECD #443) testing guidelines and under EPA good laboratory practice regulations (Agency), 1985, 1989; Cooper *et al.*, 2006; OECD, 2011b, a). Given these concerns, we performed a frequent oral dose based EOGRTS in *Sprague Dawley* rats to find out the potentially toxic effects in F0 (parental) and F1 (First generation).

MATERIALS AND METHODS

Chemicals and animals

Animals were maintained according to the standard protocol as described above in chapter 1.

Study design

By following OECD 443 an EOGRTS was performed and additional analyses at the age of puberty were also performed. The animals were scattered among five dose groups for better assessment of dose-response relations. In male rats 70 days and for female rats 14 days' exposure started before mating to cover at least one full spermatogenic cycle or two estrogenic cycles. The first control group received a daily oral dose of corn oil (50 µl) while 2nd, 3rd, 4th and 5th group received by gavage administration of furan 1, 2.5, 5 and 10 mg kg^{-1},70 days for male rats and female rats 14 days exposure (Fig 21). Dose time was chosen according to the standard protocol 443 of the OECD (2011). Different doses of furan were selected to assess the dose-dependent EOGRTS exposure-response. Furan doses used in this experiment were selected based on doses used in previous studies on furan and acrylamide with some slight modifications in concentration (Kim *et al.*, 2004; Karacaoğlu and Selmanoğlu, 2010; Rawi *et al.*, 2012; Rehman *et al.*, 2019a; Rehman *et al.*, 2019b) and OECD guideline # 408. In male rat's dose exposure continued during mating and in female dam's dose exposure continued during pregnancy and lactation. Animals were housed in couples for mating, avoiding sibling mating. Pregnant females were singly housed. Presence of sperm in vaginal smear or in the dam's vaginal plug was considered as GD 0 or day 0 of pregnancy. On GD 0, 6, 13, and 20 mated females were weighed. On GD 18 each female was shifted to a shoe-box cage. At the beginning of GD 20 females were monitored twice daily, for verification of littering. Male rats were monitored during pre-breed and mating period, and female rats during pre-breed, gestation and lactation period with a complete physical examination. Also, the number of dead and living pups, sex ratio, weights, nipple retention, anogenital distance (AGD), and survival rate were noted. Litter size was not uniform. F1 females were housed individually while F1 males had to be housed in small groups of 5-6 animals due to space limits All the reproductive parameters of F1

males were documented. F1 animals were marked for either reproductive tests around 10 weeks of age (hormonal profile, body weights and histopathology) etc. (Fig 21).

Formulas:

$$\text{Mating index (female) (\%)} = \frac{\text{No. plug/sperm positive}}{\text{No. paired}} \times 100$$

$$\text{Mating index (male) (\%)} = \frac{\text{No. males impregnating}}{\text{No. males paired}} \times 100$$

$$\text{Fertility index (\%)} = \frac{\text{No. pregnant}}{\text{No. paired}} \times 100$$

$$\text{Fecundity index (\%)} = \frac{\text{No. pregnant}}{\text{No. plug/sperm positive}} \times 100$$

$$\text{Gestational index (\%)} = \frac{\text{No. live litters, PND 0}}{\text{No. total litters, PND 0}}$$

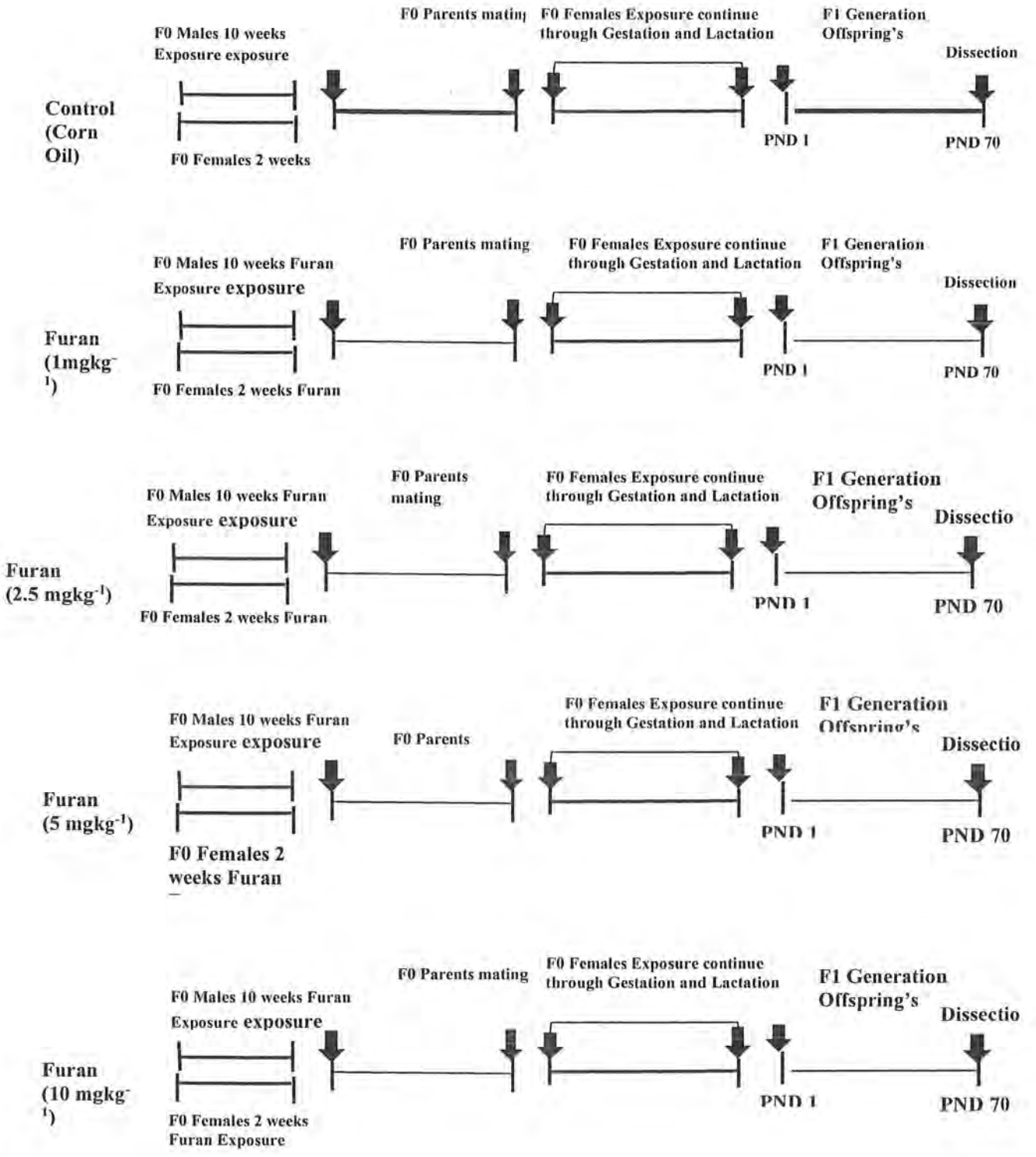


Fig 21. Schematic presentation of the generation study experiment.

Blood and tissue collection

At PND 70, male rats (n=10/group) and female rats (n=10/group) were weighed. Blood was collected. While testicular, epididymis, prostate, seminal vesicles, uterus, and ovarian organs were sampled, weighed and processed for histological and sperm parameter. At PND 70 we only proceed with the study of male rats and the samples of female rats were saved for future study. The main focus of our current study is male rat's reproductive physiology and the female investigation is out of the scope of our current investigation.

Bodyweight gain

On PND 70 animals were weighed and mean body weight gain was attained.

Determination of anogenital distance, nipple retention and puberty onset

On PND 1 the number of male and female pups were totalled and the weight of each pup was recorded. The pups were examined for deformities and in all pups, the anogenital distance was examined. From postnatal day 34, the vaginal opening (female) and preputial separation (male) of the pups (n ¼ 10/ group) was noticed every day. The day that a complete preputial separation and the vaginal opening was seen was considered as the day of puberty onset for that animal and an average day of puberty onset for each group was attained and described.

Daily sperm production (DSP)

As described in chapter 3.

Histopathology

As described in chapter 2.

Hormonal analysis

Plasma levels of T (Cat # BC-1115, Bio Check Inc., USA), FSH (RD-FSH-Ra, Reddot Biotech Inc. Canada) and LH (RD-LH-Ra, Reddot Biotech Inc. Canada) were determined by ELISA kit.

Statistical analysis

The results were stated as mean ± SEM. lme4 (Bates *et al.*, 2014) and easyanova (Arnhold, 2013) package of R 3.2.5 (R Development Core Team, 2016) were used for statistics. By using *Shapiro-Wilks*, *Levene's* and *Tukey1-dFTest* we tested the statement of variances and additivity of the class. The effect of different treatments was analysed by ANOVA ea1 command of R (post hoc *Tukey's HSD)*, While for others Chi-square test was used. $P<0.05$ considered statistically significant Value.

RESULTS

Effects of furan exposure on F0 parental body weight gains

The decrease in body weight of pre breed male (P=0.02) 5 mg kg $^{-1}$ and (P=0.001)10 mg kg $^{-1}$ and pre breed female (P=0.1) 5 mg kg $^{-1}$, (P=0.03) 10 mg kg $^{-1}$ were observed in the furan treated. Similarly, the F0 females showed significantly decreased body weight during the gestational period in the highest dose (P=0.05) 10 mg kg $^{-1}$ furan treated groups (Table 23). However, the bodyweight of F0 females significantly increased during the lactation period in 10 mg kg $^{-1}$ (P=0.02) furan treated groups (Table 23).

Table: 23. Effects of furan exposure on F0 parental body weight gain.

	Furan mgkg-^{1}					
F0	**Control**	**1**	**2.5**	**5**	**10**	**Statistics**
Prebreed (n)	30	30	30	30	30	
Males(g)	219.36±9.19^{a}	206.5±11.67^{ab}	198.83±12.3^{ac}	180.06±6.33^{bc}	169.76±5.96^{c}	P=0.001, F=4.53
Females (g)	199.16±6.75^{a}	188.96±6.57^{ab}	191.5±9.14^{ab}	176.93±4.54^{ab}	172.56±5.63^{b}	P=0.03, F=2.65
Gestation (n)	23	21	24	20	17	
Females (g)	105.13± 7.24^{a}	97.26±7.53^{a}	95.73±9.88^{a}	81.13±7.32^{ab}	67.46 ±14.09^{b}	P=0.05, F=2.36
Lactation (n)	23	21	22	18	13	
Females(g)	19.75± 2.64^{b}	23.68±4.50^{ab}	20.53±2.46^{ab}	28.25±3.64^{ab}	33.93±4.41^{a}	P=0.02, F=2.93

P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P<0.05) in the rows compared to control with other treated groups.

[a]Values are presented as mean ± SEM

[b]Significance at P<0.05 vs control

Effects of furan exposure on parental F0 head tilt and foot splay

After the observation of physical parameters, a significant difference was observed in head tilt and foot splay of F0 males. Similarly, In F0 females only significant difference was also observed in foot splay (Table 24).

Table: 24. Effects of furan exposure on parental F0 head tilt and foot splay.

	Furan mg kg^{-1}					
F0 Males	**Control**	**1**	**2.5**	**5**	**10**	**Statistics**
No. examined	30	30	30	30	30	
No. (%) with head tilt	3(10)	2 (6.66)	5(16.66)	11(33.33)	13(43.33)	P=0.04
No. (%) with foot splay	2(6.66)	2(6.66)	5(16.66)	8(26.66)	11(36.66)	P=0.02
F0 Females						
No. examined	30	30	30	30	30	
No. (%) with head tilt	3(10)	2(6.66)	1(3.33)	6(20)	7	P=0.1
No. (%) with foot splay	2(6.66)	0	3(10)	7(23.33)	9(30)	P=0.05

The data are presented as the number (and per cent) of the 30 animals per specified dose group/ sex which exhibited the finding(s) at least once from weaning to demise. ($P<0.05$) considered as significant by using Chi-Square test

Effects of furan exposure on parental F0 reproductive parameters

The reproductive parameters of F0 parental rats (to delivered F1 offspring) were shown in Table 25. The percentage of Plug/sperm-positive females (P=0.97), % age of pregnant females (66.6 % and 56.6 % respectively) and Gestational length (P=0.87) were non-significantly changed among all furan treated (Table 25). Additionally, live pups/litter was reduced in 10 mg kg^{-1} groups (P=0.001, F=4.81; Table 25).

Effects of furan exposure on F1 litters parameters during lactation

In F1 generation total number of live pups and pups born was significantly (P<0.001) reduced in furan treatment groups. There was no difference observed in sex ratio and survival of F1 pups during the lactation period. The litter size of F0 high dose dams was reduced significantly (P<0.001) during the lactation periods. Pups mean body shown in (Fig 22, Fig 23, Fig 24). AGD (Anogenital distance) was non-significantly affected by treatment in both in male and female pups. Males usually have zero nipples but sometimes a few nipples could be observed. The exposure of parents to furan did not affect the NR (nipple retention) in F1 males (P=0.07, F=2.28; Table 26).

Table: 25. Effects of furan exposure on parental F0 reproductive parameters.

	Furan (mg kg^{-1})					
	Control	**1**	**2.5**	**5**	**10**	**Statistics**
No.F0 pairs at mating (n)	30	30	30	30	30	
No. (%) Plug/sperm positive females	27(90)	25(83.33)	29(96.33)	25(83.33)	25(83.33)	P=0.97
No. (%) pregnant females	23(83.33)	21(76.66)	24(80)	20(66.66)	17(56.66)	P=0.83
No. live pups/litter (PND 0)	9.90±2.11[a]	9.81±2.09[a]	9.45±2.05[a]	6.54±1.39[ab]	5.72±1.22[b]	P=0.001 F=4.81
No. live litters on PND 0	23	21	22	19	15	P=0.73
No. live litters on PND 4	23	21	22	18	13	P=0.49
Gestational length (d)	22.1±4.71	22.0± 4.70	22.2± 4.73	22.4±4.77	22.5±4.79	P=0.87, F=0.30
Index (%)						
Fecundity index %	85.18	84	80	80	68	P=0.67
Fertility index %	76.66	70	80	66.66	56.66	P=0.31
Gestational index %	100	100	100	94.73	86.66	P=0.84

P and F value in the rows of Gestational length and No. live pups/litter (PND 0) from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. While data of number (Percentages) and index were compared by using Chi-square test. Mean with different superscript are significantly different ($P<0.05$) in the rows compared to control with other treated groups.

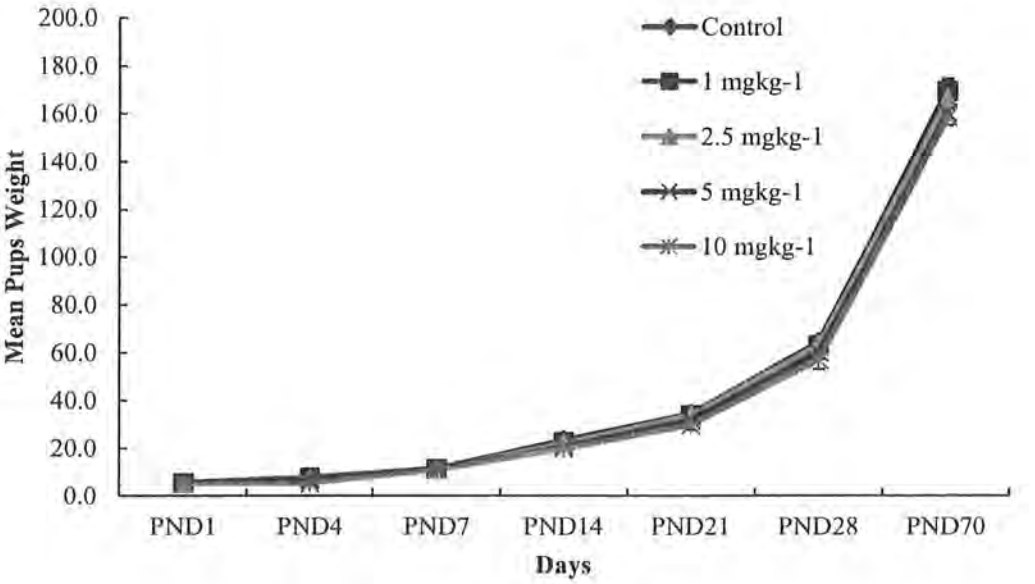


Fig 22. Mean body weights of F1 litters from PND1-PND70. Data are presented in (mean ± SEM) ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with parently furan (0, 1, 2.5, 5 & 10 $mgkg^{-1}$) exposure groups in F1 generation.

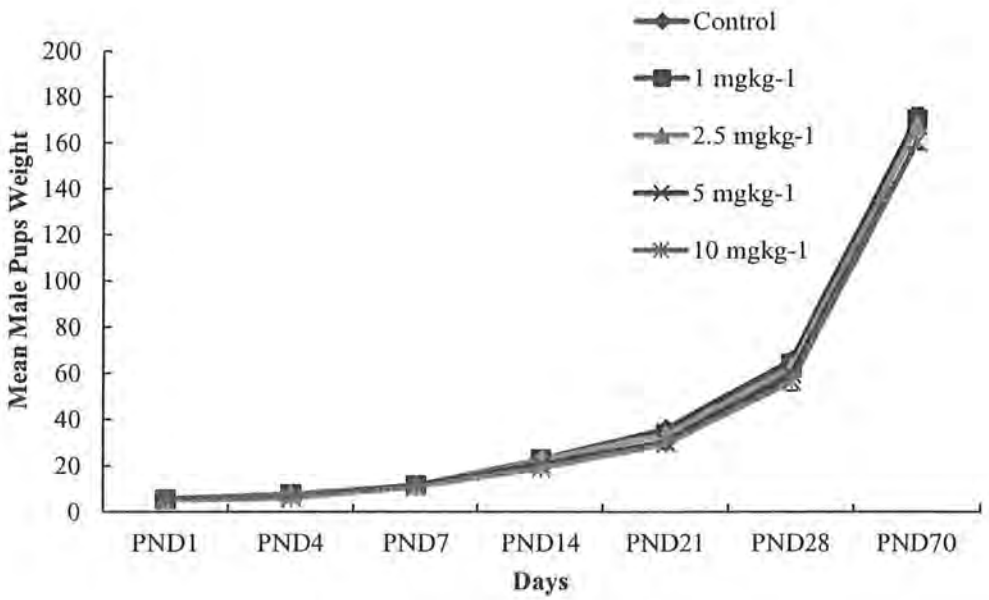


Fig 23. Male body weights of F1 litters from PND1-PND70. Data are presented in (mean ± SEM) ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with parently furan (0, 1, 2.5, 5 & 10 mg kg^{-1}) exposure groups in the F1 generation.

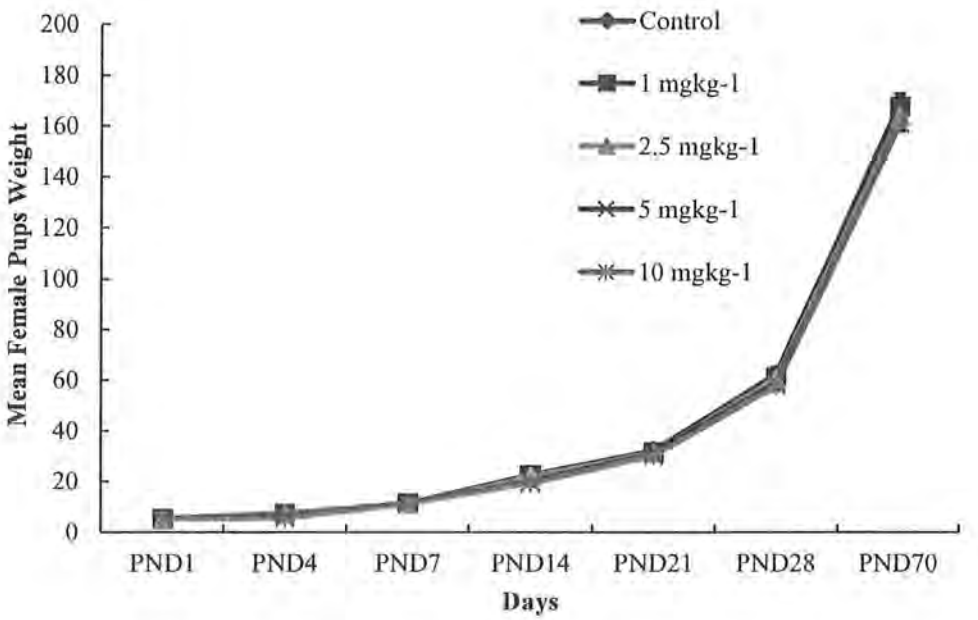


Fig 24. Female body weights of F1 litters from PND1-PND70. Data are presented in (mean ± SEM) ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with parently furan (0, 1, 2.5, 5 & 10 mg kg^{-1}) exposure groups in the F1 generation.

Table: 26. Effects of furan exposure on F1 litters parameters during lactation.

	Furan mg kg^{-1}					Statistics
	Control	**1**	**2.5**	**5**	**10**	
No. litters delivered	23	21	22	19	15	P= 0.77
No. total pups born	189	183	181	104	94	P<0.001
No. live pups (PND 0)	184	176	174	102	92	P<0.001
No. live pups (PND 4)	184	176	174	100	89	P<0.001
No. live pups (PND 7)	184	176	174	100	89	P<0.001
No. live pups (PND 14)	184	176	174	100	89	P<0.001
No. live pups (PND 21)	184	176	174	100	89	P<0.001
No. live pups (PND 28)	181	174	169	99	87	P<0.001
Sex ratio (% males, PND 0)	50.10±7.56	44.54±5.76	47.12±8.78	46.73±12.84	48.61±15.85	P=0.95, F=0.16
Live litters size (PND0)	9.57±0.16[a]	9.46±0.22[a]	9.21±0.33[a]	6.96±0.64[b]	5.87±0.35[b]	P<0.001, F=19.67
AGD in males	3.7±0.22	3.63±0.24	3.54±0.24	3.53±0.11	3.51±0.12	P=0.95, F=0.16
AGD in females	2.41±0.08	2.36±0.09	2.39±0.07	2.35±0.08	2.36±0.09	P=0.98, F=0.08
Nipple Retention (NR) in Male	0.26±0.02	0.22±0.02	0.21±0.01	0.19±0.02	0.18±0.01	P=0.07,F=2.28
Indices (%)b						
Live birth index	100	98.90±4.23	98.26±6.72	97.5±2.50	97.89±5.96	P=0.99,F=0.04
4-d survival	97.48 ±2.51	96.70±3.00	98.12±1.83	98.04±3.93	96.53±2.84	F=0.08 P=0.98
7-d survival	100 ±0.0	100 ±0.0	100 ±0.0	100 ±0.0	100 ±0.0	P=0.91, F=0.23
14-d survival	100 ±0.0	100 ±0.0	100 ±0.0	100 ±0.0	100 ±0.0	P=0.91, F=0.23
21-d survival	100 ±0.0	100 ±0.0	100 ±0.0	100 ±0.0	100 ±0.0	P=0.91, F=0.23
28-d survival	98.55±1.37	99.24±1.03	97.32±2.31	99.33±0.95	97.90±2.25	P=0.92, F=0.22
Lactation index	98.55±1.37	99.24±1.03	97.32±2.31	99.33±0.95	97.90±2.25	P=0.92, F=0.22

P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P<0.05) in the rows compared to control with other treated groups.

[a]Values are presented as mean ± SEM

[b]Significance at P<0.05 vs control

Effects of furan exposure on F1 generation at PND 70

The parental furan exposure did not affect the pup's puberty (Table. 27). On PND 70 male rats were dissected. Results of male rats indicate that the testicular weight was significantly (P=0.03) decreased in the high dose 10mgkg^{-1} treated group while there was no significant change observed in weight of prostate, seminal vesicle and epididymis in a dose-dependent manner. The DSP decreased in 5 mg kg^{-1} (P=0.04) and 10 mg kg^{-1} (P=0.01) treated groups respectively (Table. 27).

Table. 27. Effects of furan exposure on F1 generation at PND 70.

	Furan mg kg^{-1}					
	Control	**1**	**2.5**	**5**	**10**	**Statistics**
Mean pup weight (g) PND 70 (n)	170.28±3.32	169.57±5.12	166.14±3.40	160.42±4.80	158.64±3.42	P=0.16, F=1.66
Mean male pup weight (g) PND 70 (n)	171.60±3.17	170.28±5.09	167.50±2.20	160.42±3.27	160.78±3.08	P=0.07, F=2.20
Mean female pup weight (g) PND 70 (n)	169.49±3.33	167.64±5.39	164±2.21	161.14±2.81	161.50±3.56	P=0.39, F=1.02
Paired testis weight	2.97±0.08^{a}	2.82±0.17^{ab}	2.63±0.11^{ab}	2.46±0.17^{ab}	2.20±0.20^{b}	P=0.03, F=2.82
Epididymus weight	1.24±0.09	1.12±0.04	1.01±0.02	1.05±0.01	1.00±0.02	P=0.37, F=1.08
Prostrate	1.20±0.09	1.16±0.02	1.02±0.02	1.23±0.07	1.19±0.09	P=0.37, F=1.07
Semininal vesicles	0.75±0.08	0.75±0.08	0.76±0.09	0.68±0.06	0.71±0.07	P=0.97, F=0.12
DSP×10^{6}	87.6±2.48^{a}	81.2±4.18^{ab}	75±3.37^{ab}	71.4±4.36^{b}	68.8±2.93^{b}	P=0.01, F=4.03
Preputial separation	43.05±0.23	43.11±0.24	43.09±0.32	43.18±0.25	43.27±0.23	P=0.97, F=0.11

P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P<0.05) in the rows compared to control with other treated groups.

[a]Values are presented as mean ± SEM

bSignificance at P<0.05 vs control

Effects of furan exposure on F1 generation histopathology at PND 70

On PND 70, parental exposure to different concentrations of furan exhibited some obvious changes in the testis histology of F1 generation. The lumen diameter was increased in higher dose treated groups. Changes were also found in tubular diameter and epithelial height but were not statistically different (Table 28, Fig 25).

Table. 28. Effects of furan exposure on F1 generation histopathology at PND 70.

	Furan (mg kg^{-1})					
Male Testis	**Control**	**1**	**2.5**	**5**	**10**	**Statistics**
Tubular diameter	213±10.55	205±13.9	194±9.01	182±8.16	177±8.58	P=0.11, F=1.90
Lumen diameter	30±2.00[b]	32±2.84[b]	32±1.89[ab]	37±2.15[a]	39±2.88[a]	P=0.04, F=1.53
Epithelial heights	64±4.04	65±3.71	69±3.51	70±2.87	71±4.23	P=0.64, F=0.62

P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different ($P<0.05$) in the rows compared to control with other treated groups.

[a]Values are presented as mean ± SEM

[b]Significance at $P<0.05$ vs control

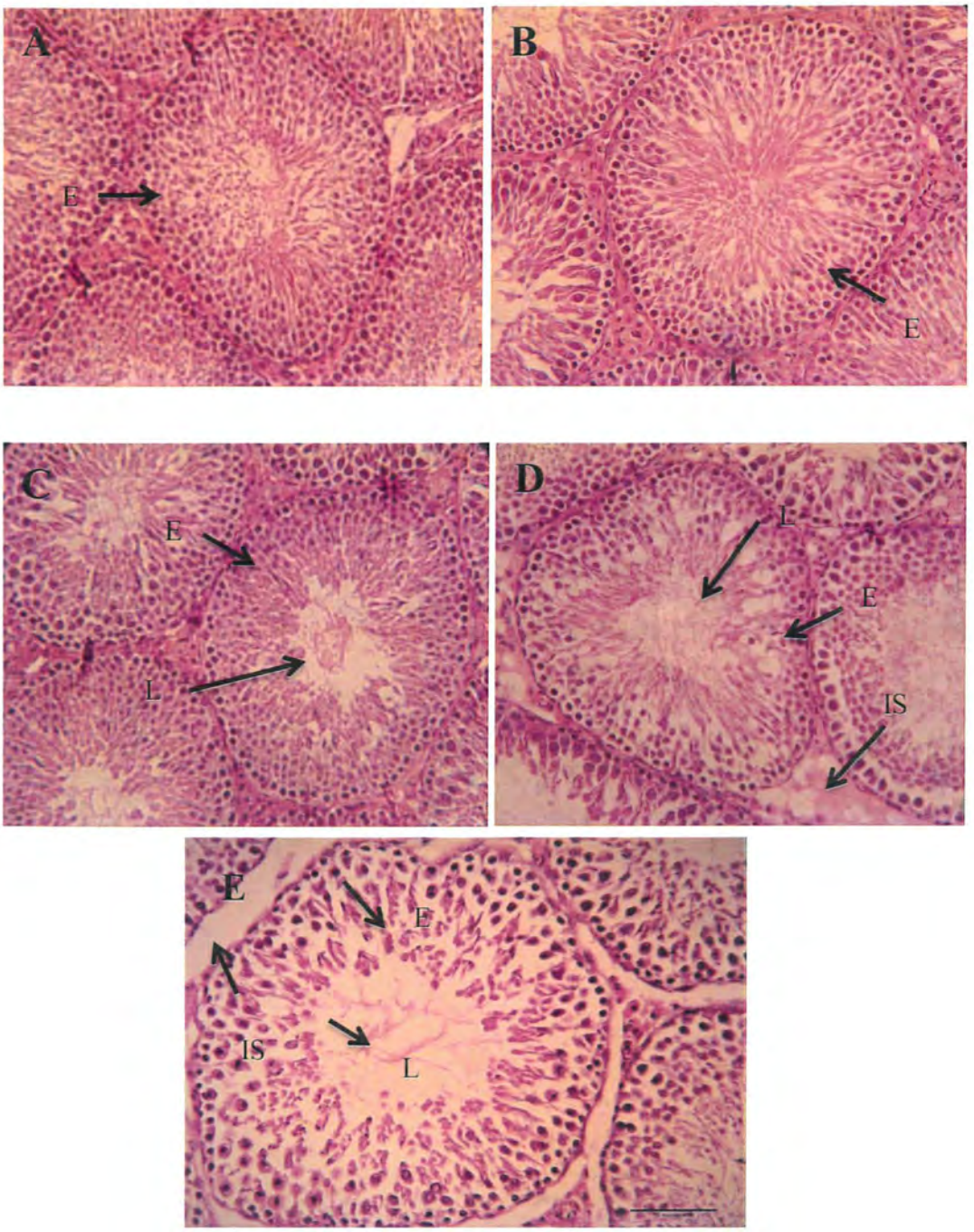


Fig. 25. Photomicrograph of a cross section of seminiferous tubules of male rats in F1 generation, with both parents receiving doses of Furan. Control; showing regular tubules (B) 1 mg kg^{-1} group; showing normal epithelial height, lumen filled with mature spermatozoa (C) 2.5 mg kg^{-1} group; normal morphology, lumen filled with immature spermatozoa, (D) 5 mg kg^{-1} group; showing increased lumen diameter and interstitial spaces, (E) 10mg kg^{-1} group; thin degenerated epithelium with the empty lumen and interstitial spaces. Lumen (L), Epithelium (E), Interstitial spaces (IS).

Effects of furan exposure on hormonal profile at PND 70

There was no statistically significant difference observed in FSH and LH concentration in 10 mg kg^{-1} furan treated male groups compared with control while the plasma T level was significantly (P=0.03) reduced in 10 mg kg^{-1} treated group (Table 29).

Table. 29. Effects of furan exposure on hormonal profile at PND 70.

			Furan mg kg^{-1}			
Male	**Control**	**1**	**2.5**	**5**	**10**	**Statistics**
T (ng ml^{-1})	6.86±0.48[a]	6.12±0.47[a]	6.00±0.33[a]	5.34±0.41[a][b]	4.44±0.43[b]	P=0.03,F=2.77
LH (ng ml^{-1})	2.33±0.13	2.31±0.13	2.30±0.13	2.04±0.17	1.80±0.17	P=0.06,F=2.34
FSH (mlUml $^{-1}$)	1.23±0.18	1.24±0.07	1.21±0.14	1.14±0.08	1.04±0.07	P=0.77,F=0.45

P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P<0.05) in the rows compared to control with other treated groups.

[a]Values are presented as mean ± SEM

[b]Significance at P<0.05 vs control

DISCUSSION

After fertilization complex physiological and endocrine mechanisms have involved that control the embryonic development. Any alteration in these mechanisms can cause many abnormal functioning in organisms. It has been previously observed that when parents were exposed to environmental toxicants that may cause developmental, behavioural and hormonal changes in the body of offspring (Tyla *et al.*, 2000; Tyl *et al.*, 2008; van der Ven *et al.*, 2009). EOGRT demonstrated that long term exposure of furan to parents resulted in parental F0 toxicity and F1 generation toxicity in both sexes at higher doses 5mg kg^{-1} and 10mg kg^{-1}. Normal development of generative systems depends upon the proper functioning of testis, ovaries, pituitary and hypothalamus. The hormonal secretions from pituitary and gonads are necessary for the development of reproductive systems.

Endocrine regulation involves the secretion of GnRH, LH and FSH, all these hormones are necessary for the normal functions of reproduction (spermatogenesis and folliculogenesis) (Amory and Bremner, 2003; Chapin *et al.*, 2008; Aritonang *et al.*, 2017). It has been previously detected that exposure to some EDCs can lead to disturbance of the several normal body functions. In rodents throughout the development period, exposure to EDCs at higher concentrations disturbed the generative functions in parenthood (Gray Jr *et al.*, 2001). Exposure to environmental toxicants e.g. acrylamide, bisphenol, tetrabromobisphenol A and imidacloprid brought variations in development and endocrine functions, thus affected F1 and F2 generations (Tyla *et al.*, 2000; Tyl *et al.*, 2008; van der Ven *et al.*, 2009; Vohra and Khera, 2016). Furan also induces chromosomal changes in Chinese hamster V79 derived cell line (NTP, 1993; Glatt *et al.*, 2005). Previously, scrutinized that different concentrations of furan induced toxic effects on the endocrine systems of animals and humans (Karacaoğlu and Selmanoğlu, 2010; Cooke *et al.*, 2014; Pandir, 2015b).

The current investigation, a significant change in F0 parental weight gain of males (pre breed period) and female (pre breed period, gestation and lactation) was observed after exposure with high doses5 and 10mg kg^{-1} of furan. Similar findings were previously

reported that reduction in weight gain of animals was observed when exposed to environmental toxicants e.g. acrylamide, furan and bisphenol etc. (Tyla *et al.*, 2000; van der Ven *et al.*, 2009; Rehman *et al.*, 2019a).

Sub-chronic furan and acrylamide exposure resulted in reduced seminal vesicle and testicular weight, along with reduced serum level of T, LH, FSH and prolactin (Kara *et al.*, 2016; Rehman *et al.*, 2019a),(Burek *et al.*, 1980). Notably, observing the body weight is important for the interpretation of the reproductive effects of toxins (Aly *et al.*, 2009). The results of the current study showed a decrease in pre breed body weight gain of F0 (parents). Explanation of the reduced body weight may be because of undernourishment during growth (Abdul-Hamid *et al.*, 2007; Wang *et al.*, 2010). It may also be due to excessive breakdown of tissue and plasma protein (Yousef and El-Demerdash, 2006). In the current study, weight gain in females during gestation period was decreased while the increase in body weight gain was noticed during the lactating period. This might be due to increased food consumption during gestation and lactation period. Metabolism of females increases during gestation and lactation period and females gain additional calories during this period due to fast metabolism (Migula, 1969). In our study reduction in gestational weight gain may be due to the reason that lactation makes greater demand of mother's body than pregnancy does (Vohra and Khera, 2016). Another possible reason is low number of implantation sites during pregnancy.

In this current study head tilt and foot, splay was also observed in F0 parents after exposure with various doses of furan. This is related to the neurotoxic effects of furan. Similar results were obtained previously in which acrylamide exposure caused head tilt and foot splay in high treatment groups and results were associated to the degeneration of the monoaminergic system of the brain (Rawi *et al.*, 2012). It was reported that acrylamide interfered with these proteins and caused hind limb weakness like foot splay, reduce grip strength etc. (Spencer and Schaumburg, 1974). The effects observed in the present study must be related to neurotoxic and reproductive toxic nature of furan.

In the current study reduction in several pregnant F0, the female was observed in higher dose treated groups. The possible reason for reduced pregnancy may be that furan generates ROS in sperm cells and causes sperm DNA damage (Pandir, 2015b). Another reason is mounting behaviour/libido failure (Tyla *et al.*, 2000). Furan treatment might affect the rat's limbs grip, disturbing mounting behaviour so that they cannot penetrate sperm into the uterus of females resulting in reduced pregnancy. Previously it is reported that human exposure to furan revealed that furan affected lymphocytes and sperm cells by producing ROS (Pandir, 2015b).

AGD development is related to reproductive development. In male and female AGD and male NR no changes were observed. In adulthood, these parameters are studies to checked the hazardous effects of toxins on reproduction (Bergkvist *et al.*, 2008; Welsh *et al.*, 2008). According to OECD protocols, these parameters are considered mandatory for testing of newly synthesized chemicals (OECD, 2013).

In our study, we observed the reduced fertility rate/number of pups in F1 generation in high ($10 mgkg^{-1}$) dose treatment group. It is well known that the total number of live pups and litter size reduction is correlated with the reduction in implantation sites (Siddiqui *et al.*, 2007). Similarly, chemical exposure suppresses the LH surge or delays preovulatory LH surge which is correlated with delayed or blocked ovulation (Dalu *et al.*, 2002; Siddiqui *et al.*, 2007). This continuous suppression of LH and delayed ovulation is associated with the significant reduction in litter size and many implantation sites that affect several live pups, fertility and mating process. Although the AGD in males and females were not significantly affected a mild decrease was observed. Reduced pups weight was observed only in PND 0 and PND 4 when the pups depend on their mother for milk. This may be due to furan exposure to the mother may cause detrimental effects in term of milk quality, quantity and may have potential toxic effects on growth retardation of the offspring. No postnatal mortality was observed in F1 generation. However, one of the main purposes for EOGRTS is to provide the awareness about chemical toxicity in parents, offspring and adulthood life stage.

It was noticed that puberty onset in males and females in the F1 generation was not affected significantly in the current study. Similarly, no difference was observed in the body weight gain on PND 70, but testicular and prostate weight were reduced dose-dependently. The previous study has stated that prostrate and testicular weights depend on sex hormones as a prostate response to androgen antagonists is well established (Kunimatsu *et al.*, 2004) and testicular dependency on androgen has also been shown (Nakayama *et al.*, 1979). It's means that exposure to furan during the sensitive development period of life badly affect the accessory organ weight. Similarly, in an early study decreased in ovarian weight was also recorded (Nah *et al.*, 2011).

The hormonal concentration i.e. T, LH and FSH in males on PND 70 is slightly affected in the F1 generation. In current finding T and LH levels were reduced in male rats that are due to furan toxicity on the reproductive system. Daily sperm production was also reduced in our current study that might be due to low T concentration. T is essential for maintaining all functions of accessory sex glands (Karacaoğlu and Selmanoğlu, 2010) and lack of T disrupt spermatogenesis (Boockfor and Blake, 1997). Our findings are supported by previous studies in which premenarcheal exposure of women with TCDD was associated with longer menstrual cycles (Eskenazi *et al.*, 2002). In mammalian reproduction FSH is the main hormone, it plays a vital role in the development of gonads, maturation at puberty and production of gamete (Delkhosh *et al.*, 2019). Estrogen and FSH concentration is important for female secondary sexual characteristics, development and proliferation of the ovarian follicles and plays a significantly important role in women's health (McLachlan *et al.*, 2006; Delkhosh *et al.*, 2019). Significant changes in testicular architecture were seen in the F1 generation as examined by histological analysis. Spermatogenesis is dependent on endocrine regulation of sex hormones (Amory and Bremner, 2003; De Gendt and Verhoeven, 2012; Aritonang *et al.*, 2017). Results from the present study confirmed that parental furan exposure generated alterations in the hypothalamic-pituitary-testicular and ovarian axis that altered folliculogenesis and spermatogenesis in the F1 generation. The parental furan exposure disturbed the levels of hormones in the F1 generation. The testicular and ovarian weights were reduced in the F1 generation at PND 70 with decreased DSP and disturbed estrous cyclicity in groups treated

with high dose (10 mg kg^{-1}) of furan. Histopathological changes were also observed in testis and ovaries of the F1 generation, whose parents were previously exposed with higher (10 mg kg^{-1}) doses of furan. Our findings highlight that parental exposure to furan can cause adverse effects on the F1generation.

CONCLUSION

Our effort demonstrates that extended one generation exposure of rats to furan at different concentration impairs their hypothalamic pituitary testicular axis, which over long-term exposure might affect generations. As we predict, an adverse effect of the food-based toxicant furan on mammals, that include detection of altered spermatogenesis. The toxicological profile of furan presented in this study supports the identification of furan as an endocrine disruptor of concern for human health. To enable environmental risk assessment more comprehensive molecular-based studies are recommended to further clarify the health hazardous effects of furan in living organisms.

GENERAL DISCUSSION

Various chemicals or toxicants have been classified as endocrine-disrupting chemicals (EDCs) because of their ability to alter animal pathophysiology. Any substance or mixture that exogenously enters in the body, make alterations in the functions of endocrine systems is called EDCs (WHO/IPCS, 2002). Nearly more than eight hundred chemicals/compounds are known as EDC because of their endocrine disruption potential (EHP, 2013). EDCs are dioxins, alkylphenols, insecticide, pesticides, herbicides, phytoestrogens, phthalates, fungicides, bisphenol A, polybrominated biphenyls, pharmaceutical drugs, polychlorinated biphenyls (PCBs) food-based acrylamide and furan, heavy metals like lead, cadmium arsenic and mercury may be included in the EDCs (Crisp *et al.*, 1998; Sharara *et al.*, 1998; Hodges *et al.*, 2000; Dickerson and Gore, 2007; Robin and Clanci, 2007; De Coster and Van Larebeke, 2012; Pandir, 2015a; Lehmann *et al.*, 2018; FDA, 2004). The EDCs performance is signified by their capacity to attach endocrine nuclear receptors (NRs) functioning as agonists or as antagonists (De Coster and Van Larebeke, 2012; Gore *et al.*, 2015).In actual, EDCs activate numerous hormone receptors (Mnif et al., 2011; Schug *et al.*, 2011). EDCs also intrude with the synthesis, transportation, metabolism and exclusion of hormones by diminishing the strength of endogenous hormones (De Coster and Van Larebeke, 2012; Gore *et al.*, 2015).EDCs direct targets the GnRH neurons (Gore, 2008a; Diamanti-Kandarakis *et al.*, 2009). Gonadotropin-releasing hormone (GnRH) control the Kiss1, EAP1, TTF1 and YY1(Johanna K.Mueller[a] and SabineHeger, 2014).

EDCs target every endocrine axis by interrupting their mode of actions (Gore *et al.*, 2015; Kabir *et al.*, 2015).EDCs may also damage the functions of hypothalamus and pituitary (De Coster and Van Larebeke, 2012; Gore *et al.*, 2015; Kabir *et al.*, 2015). Besides, pituitary expression of *Kiss1* and *GPR54* genes seems to be under the control of sex steroids (Johanna and Heger, 2014;Tena-Sempere, 2010).

Developmental stages are vulnerable when animals are exposed to EDCs during organ development (Richter *et al.*, 2007) changing the hormonal profile irreversibly (Lafuente *et al.*, 2003; Hinson and Raven, 2006; Liu *et al.*, 2010; Cooke *et al.*, 2014; Sajjad *et al.*, 2018). Exposure to EDCs during gestational and neonatal periods, bring changes in the development of associated structures, because of enduring variation in the

mechanisms of actions of hormones (Csaba, 1980; Makri *et al.*, 2004). In other organisms and humans, numerous inquiries have been performed to understand the impacts of EDCs throughout several developmental stages of life. According to the executive order (13045; 1997), issued by federal agencies in the United States, it has been implemented that identification and assessment of environmental health risks which affect children's health must be highly prioritized (EPA, 2017). Latest information indicates to exposed to EDCs, not only harm the exposed individual, but also the nest generation to the offspring of the treated individual's that is called transgenerational inheritance (Lauretta *et al.*, 2019).

Furan an EDCs which is used in several industries and also form in food items during heating systems such as coffee, sauces, soups, canned, jarred foods, infant formulas, and baby foods exerting adverse effect to human health including reproductive health (Cooke *et al.*, 2014; Alam *et al.*, 2017) (Bolger, 2009; EFSA, 2009; WHO, 2009; Bakhiya and Appel, 2010; Conti *et al.*, 2014; Cooke *et al.*, 2014; FDA, 2004). IARC categorized furan as viably humans carcinogenic substance (IARC, 1995) and EFSA described the carcinogenic and genotoxic mechanism of furan (NTP, 1993; EFSA, 2004, 2006). The FDA noticed that numerous high temperature cooked foods had measurable furan, along with the kid foods in jars and cans. The high furan was observed in mostly beans, sweet potatoes, squash, stuffed in cans (Crews and Castle, 2007). According to European Society furan were found in coffee, baby food, sauces, soups and also more in 262 out the 273 baby food products (Crews and Castle, 2007). Furan induces liver and hepatic necrosis and DNA damage, which is in turn correlated with a rise in serum alanine aminotransferase (ALT) in rats (Gill *et al.*, 2010; McDaniel *et al.*, 2012). Previously reported that the furan metabolite promotes proliferation of cell and disconnect the oxidative phosphorylation of mitochondrion (Kedderis, 1999). Bas and Pandir (2016) reported that furan exposure increases malondialdehyde (MDA) concentrations and declines glutathione-S-transferase (Livingstone), SOD, and catalase in rats. In a human study, furan exposure generated reactive oxygen species and affected the lymphocytes and sperm cells (Pandir, 2015). Thus, on a global scale, furan is of high concern because of its harmful effects on animals and humans (Hamadeh *et al.*, 2004). In the case of humans, food is the common source of toxicant exposure (Pressman *et al.*, 2017). Furan was also reported to occur in morning urine samples of humans in different concentrations (Ghosh *et al.*, 2015).

In current studies, possibly dangerous and endocrine disturbing consequences of furan were assessed via *in vitro* and *in vivo* exposure in rats. The different doses were selected according to OECD protocols. while a control group received DMSO 1%. While the animal of the first group was administered with furan (5mg/kg body weight) via oral gavage and observed for 14 days. The used doses are according to the proper scientific guidelines that fulfil the criteria of no-observed-adverse-effect-level (NOAEL) and tolerable daily intake (TDI). According to previous literature, human normal daily intake of furan is 1.23 to 2 microgram (Moro et al., 2012). The selected doses of furan at *in vitro* and *in vivo* assays were high as compared to the normal intake to check their possible toxic effects on tissue architecture and biochemical parameters. The doses were selected according to OECD Series on Testing and Assessment No. 19. And previously proved by (NTP, 1993; EFSA,2000; FDA).

Furan caused oxidative stress *in vitro* and *in vivo* further interrupted HPG and HPA axis, testicular structure and disturb the mechanism of spermatogenesis in subchronic, neonatal study and the possible effects of furan on F0 (parents) reproduction, and F1(first generation) offspring growth and development, with functional calculations of endocrine function following exposure during the early period of development in EOGRTS extended one-generation study.

In the *in vitro* experiment, direct effects of furan exposure on oxidative stress, ROS accumulation, lipid peroxidation, DNA damage of testicular and spermatozoa tissues were observed. The current outcomes of *in vitro* study showed decreased levels of CAT, SOD, and POD in testicular tissue. While the ROS and LPO levels were elevated in testicular tissues by furan treatment compared to control. These results are similar to previous studies in which furan and acrylamide exposure elevate the level of LPO and ROS (Mehri *et al.*, 2012; Zhao *et al.*, 2013; Pandir, 2015a). All these changes may be due to high ROS production oxygen-containing molecules that have free, unpaired electrons and are highly reactive. High ROS production imparts damaging effects on protein, lipids, and DNA (Radak *et al.*, 1999). Self-defence mechanism (antioxidant enzyme) is activated against ROS, which further decline of ROS (Kaul and Forman, 2000). The decrease in the antioxidant enzyme levels devastates the cell's sensitivity to harbour oxidative stress, hence compromising its ability to detoxify the effects of ROS (Kaul and Forman, 2000; Pérez 2009). Previously, furan was found to

be a cytotoxic, genotoxic and an apoptotic inducer (Heppner and Schlatter, 2007; Karacaoğlu and Selmanoğlu, 2010b).

In the *in vitro* study T concentration decreased in groups exposed to high doses of furan. However, a decreased level of T is the indicator of chemical toxicity (Yoshida *et al.*, 2002). As previously described, reduced T concentration is due to oxidative stress, which is connected to the main action of antioxidant enzymes within Leydig cells and enhances the sensitivity of spermatogenesis toward oxidative stress (Cao *et al.*, 2004; Rezvanfar *et al.*, 2013). From the present study, it is concluded that furan exerts its effects by induction of oxidative stress that also alters T secretion in testis, resulting in decreased sperm production and spermatogenic arrest. Similar findings were previously reported that ROS production in the cells induces cytotoxicity and cell damage (Pandir, 2015b).In rats and rabbit, ROS generation is responsible for the suppression of sperm movement and sperm loss by spermatozoa leaking electrons from the mitochondrial electron chain, resulting in the production of ROS (Vernet *et al.*, 2001; Aitken and Baker, 2004).In the sperm plasma membrane, high concentration of fatty acids is present (Aitken *et al.*, 1995) and higher ROS production causes damage to membrane lipids, disturbing sperm production and functions (Aten *et al.*, 1992; Ichikawa *et al.*, 1999; Zalata *et al.*, 2004). Our current findings from "*in vitro*" approached have shown spermatozoa incubation with furan decreased the antioxidant enzyme activity of cells. while ROS and LPO levels were increased in sperm cells. Previously it has been reported that acrylamide induces ROS production and lipid peroxidation in PC12 cells in animal models (Yousef and El-Demerdash, 2006; Cao *et al.*, 2008; Rodríguez-Ramiro *et al.*, 2011a; Rodríguez-Ramiro *et al.*, 2011b; LoPachin and Gavin, 2015).In the current study, sperm DNA damage was seen in a group exposed to a high dose of furan which might be due to the elevated level of ROS and disturbed redox balance. These findings are reliable with the previously reported work in which dose-dependent increase in comet tail, length and of the moment was observed in human lymphocytes and spermatozoa cells in *in vitro* study (Pandir, 2015b).

In the second *in vivo* experiment, research was created to examine the consequence of furan on brain monoamines levels and reproductive functions. Dose time was chosen according to the standard protocol 407 of the OECD (2008). Different doses of furan were selected to assess the dose-dependent sub-chronic exposure-

response. High doses of furan, i.e., 20 and 40 mg kg^{-1}, produce more ROS and oxidative damage testicular tissues by reducing the seminiferous tubular lumen diameter, epithelial height and decreasing the number of sperm. All these alterations might be due to high ROS production. High ROS production imparts damaging effects on protein, lipids, and DNA (Radak *et al.*, 1999)(Kaul and Forman, 2000). In current observation, plasma total cholesterol, triglyceride, and LDL levels were elevated in furan-treated animals, while the HDL level was reduced. According to previous literature, furan and acrylamide administration causes an increase in plasma LDL, total cholesterol, triglycerides and decrease in HDL (Ghanayem *et al.*, 2010; Raju *et al.*, 2015). Previously, reported that the association of increased cholesterol and reduced HDL levels is linked to male infertility, low T level, and sperm abnormality (Shalaby *et al.*, 2004).

In the *in vivo* sub-chronic study, a clear decrease was observed in sperm count. Parallel results were also previously described by (Uzunhisarcikli *et al.*, 2007), where sperm count reduced as a result of a reduction in T level. Spermatozoa exposed to the high concentration of ROS resulted in reduced sperm viability and motility, leading to infertility (Aitken and Curry, 2011) as reproductive hormones control spermatogenesis and cell-to-cell contact within the testis.In comparison with the control group. Reproductive hormones control the spermatogenesis and cell to cell contact within the testis. Degraded LH levels caused a drop in T while decreased FSH affect spermatogenesis. It has been previously reported that increased cortisol and oxidative stress decreased reproductive hormonal level (Glenn *et al.*, 2011). A decrease in the T production in adulthood is a recurrent finding in studies exposing rats to heat-induced food toxicants (Salian *et al.*, 2009; Karacaoğlu and Selmanoğlu, 2010b; El-Akabawy and El-Sherif, 2016).

Increased cortisol secretion has been associated with a decreased production of sex steroids and GH in a multitude of studies (Burguera *et al.*, 1990; Björntorp, 1995; Chen *et al.*, 1997; Tsigos and Chrousos, 2002; Viau, 2002; Liening and Josephs, 2010). Glenn, (2011) also reported that cortisol also inhibits HPG axis activity at all levels. Similar findings were observed in the current investigation that cortisol was increased, and the level of reproductive hormones was reduced. Endocrine disruptors such as PAHs and PCBs, as well as lead and cadmium cause, elevated cortisol level in fish and

mammals (Tort *et al.*, 1996; Tan *et al.*, 2007; Zimmer *et al.*, 2009; Sajjad *et al.*, 2018). The reduction in T concentration due to the anti-androgenic nature of furan as described earlier (Karacaoğlu and Selmanoğlu, 2010b; Cooke *et al.*, 2014; Pandir, 2015a). The relationships we observed between plasma hormone levels furthermore fit well in with earlier studies reporting negative relationships between plasma cortisol vs. T (Wennink *et al.*, 1990). The HPT and HPA axis, also cause the regulation of various reproductive functions, for example, HPA-axis control the action of the HPG-axis (Dobson *et al.*, 2003). In the present study, T concentration decreased in groups exposed to high doses of furan. However, a decreased level of T is the indicator of chemical toxicity (Yoshida *et al.*, 2002). As previously described, reduced T concentration is due to oxidative stress, which is connected to the main action of antioxidant enzymes within Leydig cells and enhances the sensitivity of spermatogenesis toward oxidative stress (Cao *et al.*, 2004; Rezvanfar *et al.*, 2013).

Environmental toxicants and EDCs shown to employ neurotoxic properties and interrupt the synthesis, transportation, and release of serotonin, dopamine and glutamate norepinephrine Similar findings were previously reported that various chemicals, i.e., EDCs and non-EDCs, play a synergistic and additive neurotoxic effect (Schug *et al.*, 2015). Ali, *et al* (1983) suggested that single and frequent dosages of food-based toxicant acrylamide caused high levels of 5-HT in the striatum, hippocampus, frontal cortex, hypothalamus, and brain-stem. Numerous studies specify that Pb and Cd marked the action of the monoaminergic activities during the development of CNS (Antonio *et al.*, 1996; Bressler and Goldstein, 1991; Antonio *et al.*, 1999,1998; Antonio *et al.*, 2002; Mejia *et al.*, 1997).

In the sub-chronic study, the increasing amount of DOPAC/DA and HVA /DA, and NE against chemical exposure and their correlation with cortisol influence the monoamines regulation of HPA-axis. In numerous studies, correlation of brain 5-HIAA/5-HT levels with cortisol suggested that 5-HT of the brain is involved in the regulation of HPI and HPA axis (Dinan, 1996; Winberg and Lepage, 1998; Winberg et al., 1997; Hoglund *et al.*, 2000; Heisler *et al.*, 2007). The 5HT play role in the regulation of the HPA axis activity (Curtis and Patel, 2008). In the regulation of stress reactions, the serotonergic and dopaminergic activity of the brain plays a vital role (Winberg and Nilsson, 1993; Winberg *et al.*, 2001; Bowman *et al.*, 2002; Larson *et al.*,

2003; Perreault *et al.*, 2003; Lepage *et al.*, 2005; Gesto *et al.*, 2013), which is facilitated by a various environmental stressor (Gesto *et al.*, 2008, 2009; Schjolden *et al.*, 2006). Fish species and mammals showed an elevated level of brain 5-HIAA and 5-HIAA/5-HT in response to a stressor (Alanärä *et al.*, 1998; Øverli *et al.*, 2001; Bowman *et al.*, 2002; Gesto *et al.*, 2013).

It has been previously observed that neurotransmitters such as monoamines, peptides and amino acids have a role in the neuroendocrine control mechanism of reproduction in a living organism (Gallo, 1980; Nock and Feder, 1981). The mammalian, serotonin plays a key role in the functions of reproduction i.e., socio-sexual behaviors, secretion of GnRH, gonadotropin and. gonadal maturation. Previously reported that the endocrine signaling pathways of reproduction and serotonin are closely associated with each other (Prasad *et al.*, 2015). The neuroendocrine regulates the mechanism of reproduction through the HPG axis that conserved in vertebrates (Prasad *et al.*, 2015). The 5HT is associated with gonadotropins regulation, as the 5HT neurons stimulate gonadotropin release through receptors of 5HT in the median eminence region (Spinelli, 2000). The LH level in the current study in high doses of furan that might be the regulation of the elevated level of serotonin, Similar finding were reported by Rubinow and Schmidt (2006) that 5HT axons also terminate in the neurons of the luteinizing releasing hormone, located in the preoptic area.

In the neonatal study rats exposed to furan at the neonatal stage of life and estimated the effects on the male reproductive system in adulthood. Neonatal male rats were subcutaneously injected with four different furan doses (1, 5, 10 and 20 mg kg^{-1}in 50 µL corn oil), to investigate the effects in adult male rats (PND 80). The doses were selected according to previous exposure studies with modifications (Kim *et al.,* 2004; Karacaoğlu and Selmanoğlu, 2010b; Rawi *et al.,* 2012). Subcutaneous routes of furan administered were followed as neonatal rodents have low liver enzymatic activity, therefore there is no difference between oral and non-oral administration (Chapin *et al.,* 2008; Taylor *et al.,* 2008). The doses were selected according to previous exposure studies with modifications (Kim *et al.,* 2004; Karacaoğlu and Selmanoğlu, 2010b; Rawi *et al.,* 2012). Subcutaneous routes of furan administered were followed as neonatal rodents have low liver enzymatic activity, therefore there is no difference between oral

and non-oral administration (Chapin *et al.*, 2008; Taylor *et al.*, 2008). We find clear evidence that exposure to 10 and 20 mg kg^{-1} d^{-1} of furan, broadly and consistently affects the development of the male reproductive system, ultimately compromising male fertility in adulthood. The most prominent effects we observed were dose-dependent increases in body weight, decreased plasma T, LH and GH, increased cortisol levels, and decreased sperm counts associated with altered testicular and epididymal histology. Elevated weight gain has been observed in an earlier study where rats were exposed to furan from the weaning period to post-puberty (Karacaoğlu and Selmanoğlu, 2010a) and in suckling mice after furan treatment (Blagburn *et al.*, 1998). (Mårin *et al.*, 1992) has suggested that cortisol can cause obesity also low T levels are associated with adiposity resulting from metabolic impairments (Fui *et al.*, 2014). Rasmussen (2010) reported that GH deficiency is related to increased fat mass and abdominal fat mass accumulation (Rasmussen, 2010). Hence, the increase in weight gain and abdominal fat mass we found in the present study the group treated with a higher dose (20 mg kg^{-1} d^{-1}) caused by the elevation of cortisol levels while decreased the level of T, LH and GH.

The relationships we observed between plasma hormone levels furthermore fit well in with earlier studies reporting negative relationships between plasma cortisol vs. T or GH (Wennink *et al.*, 1990). Indeed, many components of the gonadal axis are downregulated by plasma glucocorticoids, either by affecting hypothalamus and pituitary functions or by affecting the responsiveness of target tissues to gonadal hormones (Thakore and Dinan, 1994; Borges *et al.*, 1997). Increased cortisol secretion has been associated with a decreased production of sex steroids and GH in a multitude of studies (Burguera *et al.*, 1990; Björntorp, 1995; Chen *et al.*, 1997; Tsigos and Chrousos, 2002; Viau, 2002; Liening and Josephs, 2010). High doses of glucocorticoids inhibited testicular Leydig cell function in rats (BAMBINO and HSUEH, 1981).In humans, exposure to cortisol caused a marked decrease in T production (Cumming *et al.*, 1983). A decrease in the T production in adulthood is a recurrent finding in studies exposing rats to heat-induced food toxicants (Salian *et al.*, 2009; Karacaoğlu and Selmanoğlu, 2010b; El-Akabawy and El-Sherif, 2016). Also, endocrine disruptors such as PAHs and PCBs, as well as lead and cadmium cause elevated cortisol levels and decreased GH levels in fish and mammals (Tort *et al.*, 1996; Tan *et al.*, 2007; Zimmer *et al.*, 2009; Sajjad *et al.*, 2018). This suggests that the growth of the reproductive

organs can be disturbed by reduced T, which in turn could be driven by increased cortisol levels.

In the neonatal exposure study, increasing levels of DOPAC/DA, HVA /DA and NE level against to furan exposure as well as their positive co-relation with cortisol showed that monoamines influence the regulatory mechanism of HPA-axis. Similarly, cortisol is used as the main indicator of stress. In many earlier studies, it is also reported that the procedures of environmental chemicals that induce neurotoxicity and their relation to generating NO and ROS (Chattopadhyay *et al.*, 2002a;b; García Chávez *et al.*, 2003; Rao and Avani, 2004), and cortisol level (Wang *et al.*, 2018). In fact, hyperactivity of the HPA axis may damage monoaminergic neurons (Wang *et al.*, 2018).

In previous findings the brain ratios have been correlated with plasma cortisol, suggesting that 5-HT play a key role (Winberg *et al.*, 1997; Winberg and Lepage, 1998; Hoglund *et al.*, 2000) and hypothalamus pituitary adrenal (HPA) axes (Dinan, 1996; Heisler *et al.*, 2007). Curtis and Patel, (2008) also reported that 5HT regulate the activity of the HPA axis. It has been previously reported that monoamine, peptides and amino acids play an important role in the reproductive neuroendocrine control mechanism (Gallo, 1980; Nock and Feder, 1981). In the current study, the reproduction might be regulated by the neurotransmitters i.e., monoamine. Specifically, serotonin is involved in regulating the functions of reproduction. The LH level in the current study in high doses of furan that might be the regulation of the elevated level of serotonin, Similar finding have been reported by Rubinow and Schmidt, (2006).

We found a consistent decrease in both the quality and number of sperm from males neonatal exposed to the two highest doses of furan. A significant decrease in viability and motility of sperm was observed in the highest dose treated group, and daily sperm production and epididymis (caput, corpus and cauda) sperm counts were reduced. These findings may be explained by the lower T and LH concentrations. The elevated cortisol levels may have contributed to the inhibition of spermatogenesis, disturbance of spermiation and impairment of sperm quality (Castranova *et al.*, 2005; Pressman *et al.*, 2018). Also, furan exposure at the weaning stage of life resulted in

spermatogenic impairment and apoptosis in Leydig and germ cells, which is associated with lower LH and T levels (Karacaoğlu and Selmanoğlu, 2010b).

Histological analysis of groups receiving the highest doses, prominent multilayered spermatogonia were recorded, while seminiferous tubules lumen was not heavily packed with elongated spermatozoa. These findings are in accordance with earlier reports of germ and Leydig cells apoptosis after furan exposure (Karacaoğlu and Selmanoğlu, 2010b).

We observed increases in plasma cholesterol and LDL while triglyceride (TG) and HDL were decreased in the treatments receiving elevated doses of furan. These findings are in line with the observed increases in body mass and abdominal fat. The increased production of fatty acids leads to a reduction in plasma HDL and elevation in plasma cholesterol concentrations, which can ultimately result in liver dysfunction. Lipoproteins (HDL and LDL) are therefore considered sensitive biomarkers of liver function (Rawi *et al.*, 2012). TG/HDL ratio is clinically used for the detection of apparently healthy individuals with cardiovascular or metabolic impairments (Murguía-Romero *et al.*, 2013). The lowest levels of HDL are detected in patients with fasting chylomicronæmia and hypertriglyceridemic subjects, suggesting an inverse relationship between the metabolism of triglyceride and HDL (Schaefer *et al.*, 1978). However, the present study detected a decrease in both triglycerides and HDL associated with furan treatment, which could be explained by the observation that plasma lipoprotein levels often remain low during the early stages of development (Robinson and Seakins, 1963). An alternative explanation for the observed changes in plasma lipoproteins stems from the observation that several liver toxins have been reported to inhibit the release of hepatic triglyceride into the bloodstream. Acrylamide, for example, also causes changes in lipoproteins in the same direction (Rawi *et al.*, 2012). This would explain the observed low plasma triglyceride concentration and could also have led to the buildup of triglyceride in the liver (Robinson, 1973), which has been associated with liver damage and fibrosis in obese mice (Yamaguchi *et al.*, 2007). A third possible explanation for the observed elevated plasma LDL levels could be the overactivation of LDL receptors, as first proposed by Selmanoglu *et al.* (2012).In the latter study male rats were exposed to furan, which also resulted in elevated plasma LDL levels. LDL receptors assist cholesterol molecules to enter normal body cells. The

LDL release their cholesterol and triglycerides on attachment to their receptors on the hepatocytes. Elevated cholesterol decreases the new LDL receptors formation, resulting in decreased transport of LDL cholesterol into the cells (Elaine, 2009). Excess levels of free cholesterol inhibit cholesterol and LDL receptor synthesis, thus reducing LDL uptake which promotes cholesterol storage. Restricting the LDL uptake and non-functioning of its receptors enhance the serum cholesterol levels (Linda, 2012). Importantly, we observed a dose-dependent decline in fertility (number of pups conceived) in rats neonatally exposed to furan. This finding can be adequately explained by the lower T levels that arguably resulted in lower sperm numbers and quality, which were produced by testes showing clear histological abno rmalities. We find no evidence that furan exposure caused a delay in the onset of puberty (as determined from preputial separation), which has been observed for ethynylestradiol, among other EDCs (Yoshimura *et al.*, 2005).

It has been previously observed that when parents were exposed to environmental toxicants that may cause developmental, behavioural and hormonal changes in the body of offspring (Tyla *et al.*, 2000; Tyl *et al.*, 2008; van der Ven *et al.*, 2009). In the present study, the potential effects of furan on rats were evaluated in an EOGRTs and demonstrated that long term exposure of furan to parents resulted in parental F0 toxicity and F1 generation toxicity at higher doses 5mg kg^{-1} and 10mg kg^{-1}. Dose time was chosen according to the standard protocol 443 of the OECD (2011). Different doses of furan were selected to assess the dose-dependent EOGRTS exposure-response. Furan doses used in this experiment were selected based on doses used in previous studies on furan and acrylamide with some slight modifications in concentration (Kim *et al.*, 2004; Karacaoğlu and Selmanoğlu, 2010; Rawi *et al.*, 2012; Rehman *et al.*, 2019a; Rehman *et al.*, 2019b) and OECD guideline # 408. A significant change in F0 parental weight gain of males (pre breed period) and female (pre breed period, gestation and lactation) was observed after exposure with high doses 5 and 10mg kg^{-1} of furan. Similar findings were previously reported that reduction in weight gain of animals was observed when exposed to environmental toxicants e.g. acrylamide, furan and bisphenol etc. (Tyla *et al.*, 2000; van der Ven *et al.*, 2009; Rehman *et al.*, 2019). The furan toxicity at parental stage F0 in the current study is similar according to the previous findings. Sub-chronic furan and acrylamide exposure resulted in reduced seminal vesicle and testicular weight, along with reduced serum

level of T, LH, FSH and prolactin (Kara *et al.*, 2016; Rehman *et al.*, 2019). The body mass is considered the indicator of xenobiotics effect. Notably, observing the body weight is important for the interpretation of the reproductive effects of toxins (Aly *et al.*, 2009). The current study presented the decrease in pre breed body weight gain of F0 (parents). Explanation of the reduced body weight may be deficiencies in growth and protein due to malnutrition (Abdul-Hamid *et al.*, 2007; Wang *et al.*, 2010). It may also be due to excessive breakdown of tissue and plasma protein (Yousef and El-Demerdash, 2006). Weight gain in females during gestation period was decreased while the increase in body weight gain was noticed during the lactating period. This might be due to increased food consumption during gestation and lactation period. Metabolism of females increases during gestation and lactation period and females gain additional calories during this period due to fast metabolism (Migula, 1969). In our study reduction in gestational weight gain may be due to the reason that lactation makes greater demand for mother's body than pregnancy does (Vohra and Khera, 2016). Another possible reason is the low number of implantation sites during pregnancy.

In this current study head tilt and foot, splay was also observed in F0 parents after exposure with various doses of furan. This is related to the neurotoxic effects of furan. Similar results were obtained previously in which acrylamide exposure caused head tilt and foot splay in high treatment groups related to the brain monoaminergic system degeneration (Rawi *et al.*, 2012). It was reported that acrylamide interfered with the Spindle fibre proteins present in peripheral axonoal region further caused hind limb weakness like foot splay, reduce grip strength etc. (Spencer and Schaumburg, 1974). The effects observed in the present study must be related to neurotoxic and reproductive toxic nature of furan.

In the current study reduction in several pregnant F0, female was observed in higher dose treated groups. The possible reason for reduced pregnancy may be that furan generates ROS in sperm cells and causes sperm DNA damage (Pandir, 2015a). Another reason is mounting behaviour/libido failure (Tyla *et al.*, 2000). Furan treatment might affect the rat's limbs grip, disturbing mounting behaviour so that they cannot penetrate sperm into the uterus of females resulting in reduced pregnancy. Previously it is reported that human exposure to furan revealed that furan affected lymphocytes and sperm cells by producing ROS (Pandir, 2015a).

In our study, we observed the reduced fertility rate/number of pups in F1 generation in high ($10mgkg^{-1}$) dose treatment group. It is well known that the total number of live pups and litter size reduction is correlated with the reduction in implantation sites (Siddiqui *et al.*, 2007). Similarly, chemical exposure suppresses the LH surge or delays preovulatory LH surge which is correlated with delayed or blocked ovulation (Dalu *et al.*, 2002; Siddiqui *et al.*, 2007). This continuous suppression of LH and delayed ovulation is associated with the significant reduction in litter size and several implantation sites that affect the number of live pups, fertility and mating process. Although the AGD in males and females were not significantly affected a mild decrease was observed. Reduced pups' weight was observed only in PND 0 and PND 4 when the pups depend on their mother for milk. This may be due to furan exposure to the mother may cause detrimental effects in term of milk quality, quantity and may have potential toxic effects on growth retardation of the offspring. No postnatal mortality was observed in F1 generation. The above data of F0 is closely related to the previous observation of reproductive toxicity of acrylamide and furan (Karacaoğlu and Selmanoğlu, 2010b; Cooke *et al.*, 2014; Rehman *et al.*, 2019).

It was noticed that puberty onset in males in the F1 generation was not affected significantly in the current study. Similarly, no difference was observed in the body weight gain on PND 70, but testicular and prostate weight were reduced dose-dependently. The previous study has stated that prostrate and testicular weights depend on sex hormones as a prostrate response to androgen antagonists is well established (Kunimatsu *et al.*, 2004) and testicular dependency on androgen has also been shown (Nakayama *et al.*, 1979). It's means that exposure to furan during the sensitive development period of life badly affect the accessory organ weight. Similarly, in an early study decreased in ovarian weight was also recorded (Nah *et al.*, 2011). The hormonal concentration i.e. T, LH and FSH in males on PND 70 is slightly affected in the F1 generation. In current finding T and LH levels were reduced in male rats that are due to furan toxicity on the reproductive system. Daily sperm production was also reduced in our current study that might be due to low T concentration. T is essential for maintaining all functions of accessory sex glands (Karacaoğlu and Selmanoğlu, 2010b) and lack of T disrupt spermatogenesis (Boockfor and Blake, 1997). Our findings are supported by previous studies in which premenarcheal exposure of women with TCDD was associated with longer menstrual cycles (Eskenazi *et al.*, 2002). In

mammalian reproduction FSH is the main hormone, it plays a vital role in the development of gonads, puberty maturation and production of gamete during the fertile period (Delkhosh *et al.*, 2019). Estrogen and FSH concentration is important for female secondary sexual characteristics, development and proliferation of the ovarian follicles and play a significantly important role in women's health (McLachlan *et al.*, 2006; Delkhosh *et al.*, 2019). Significant changes in testicular architecture were seen in F1 generation as examined by histological analysis. Spermatogenesis are dependent on endocrine regulation of sex hormones (Amory and Bremner, 2003; De Gendt and Verhoeven, 2012; Aritonang *et al.*, 2017).

The findings of the present study revealed that parental furan exposure generated alterations in the hypothalamic-pituitary-testicular axis that altered spermatogenesis in the F1 generation. The parental furan exposure disturbed the levels of T, LH and FSH in male rats in the F1 generation. The testicular weights were reduced in the F1 generation at PND 70 with decreased DSP and disturbed estrous cyclicity in groups treated with high dose (10 mg kg^{-1}) of furan. Histopathological changes were also observed in the testis of the F1 generation, whose parents were previously exposed to higher (10 mg kg^{-1}) doses of furan. Our findings are in line with previous observations and findings on furan exposure highlight that parental exposure to furan can cause adverse effects on the F1generation.

GENERAL CONCLUSIONS

Current experimentations proved that exposure to furan affects the reproductive functions and physiology in rats. It is concluded that.

- In the *in vitro* study, furan exposure showed increased genotoxic and oxidative stress-producing potential. which is due to ROS and LPO generation in testicular tissues and epididymal sperm cell counts.
- Loss of cell viability is related to the initiation of sperm DNA damage which was observed in furan exposure *in vitro* study.
- Fluctuation in testosterone, gonadotrophins, cortisol concentrations and brain monoaminergic activity were more prominent in furan exposure groups, suggesting that it can affect fertility by interfering HPG and HPA axis.
- Exposure to furan during developmental can cause organizational effects on the development of reproductive systems. This study provides important preclinical data on the minimal furan dose at which endocrine disruption can occur.
- In extended one-generation reproductive toxicity study (EOGRTs), intended adverse effects were observed in parental (F0) reproductive parameters and first-generation (F1) reproductive parameters of rats after furan exposure. It can be concluded that the furan has potential health hazardous effects on the rat's reproductive system

From these findings, it is concluded that furan may be a matter of high concern for human life and health. In summary, the current findings study is in line with FDA findings. The current dataset will help to understand the effects of furan and its impacts on human health.

FUTURE PERSPECTIVES

In the present study, detailed reproductive toxicity of furan has been well investigated using rats. However, further investigations are needed to fulfil the consequences/gaps regarding furan toxicity.

- The high demand of ready to eat; studies should be conducted in occupationally exposed humans to find out the exact mechanism of abnormalities in humans.
- Metabolic derivatives of furan need to be evaluated.
- Further molecular-based investigation is needed to recognize the actual mechanism of action at the cellular level.
- Another aspect is to find out the effect of furan on sexual and anxiety-like behavioural responses in male and female's rats.
- There is a need to study the interaction of furan with other cellular receptors should be determined.
- There is a need to understand the mechanism of gene expression in reproductive organs after furan exposure.

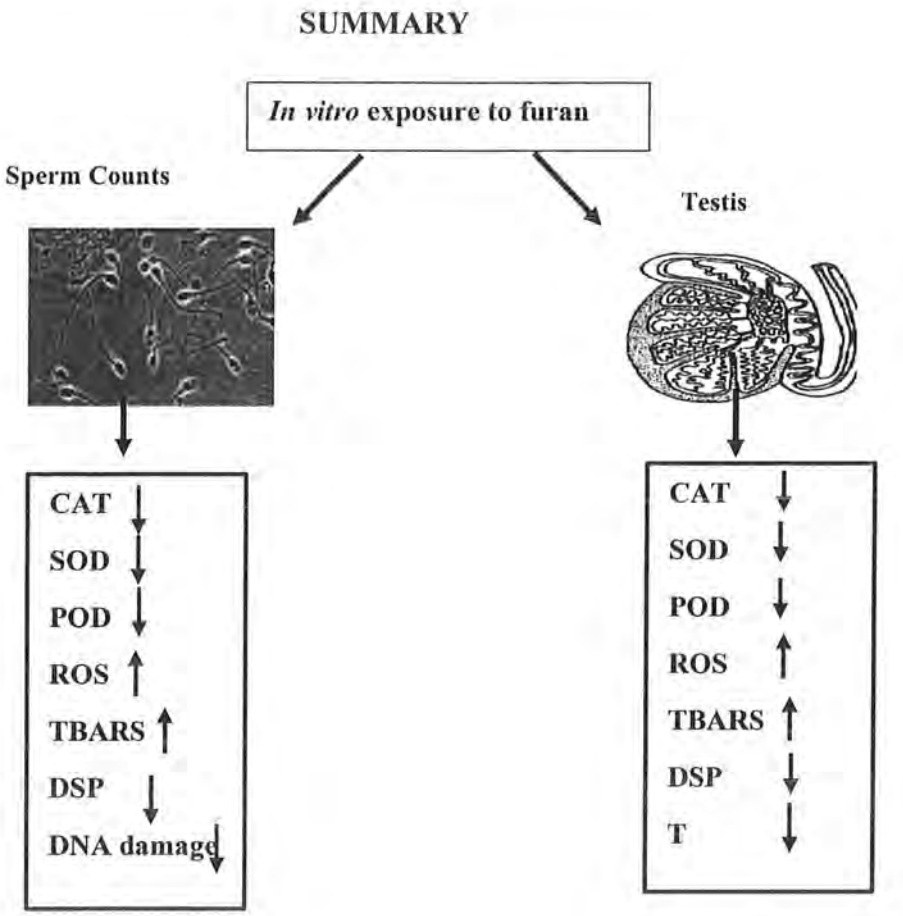


Fig 26. Schematic representation of the effect of *in vitro* furan exposure on sperm and testis of rats. Exposure to Furan can induce oxidative stress in the testis and sperm and can reduce T secretion.

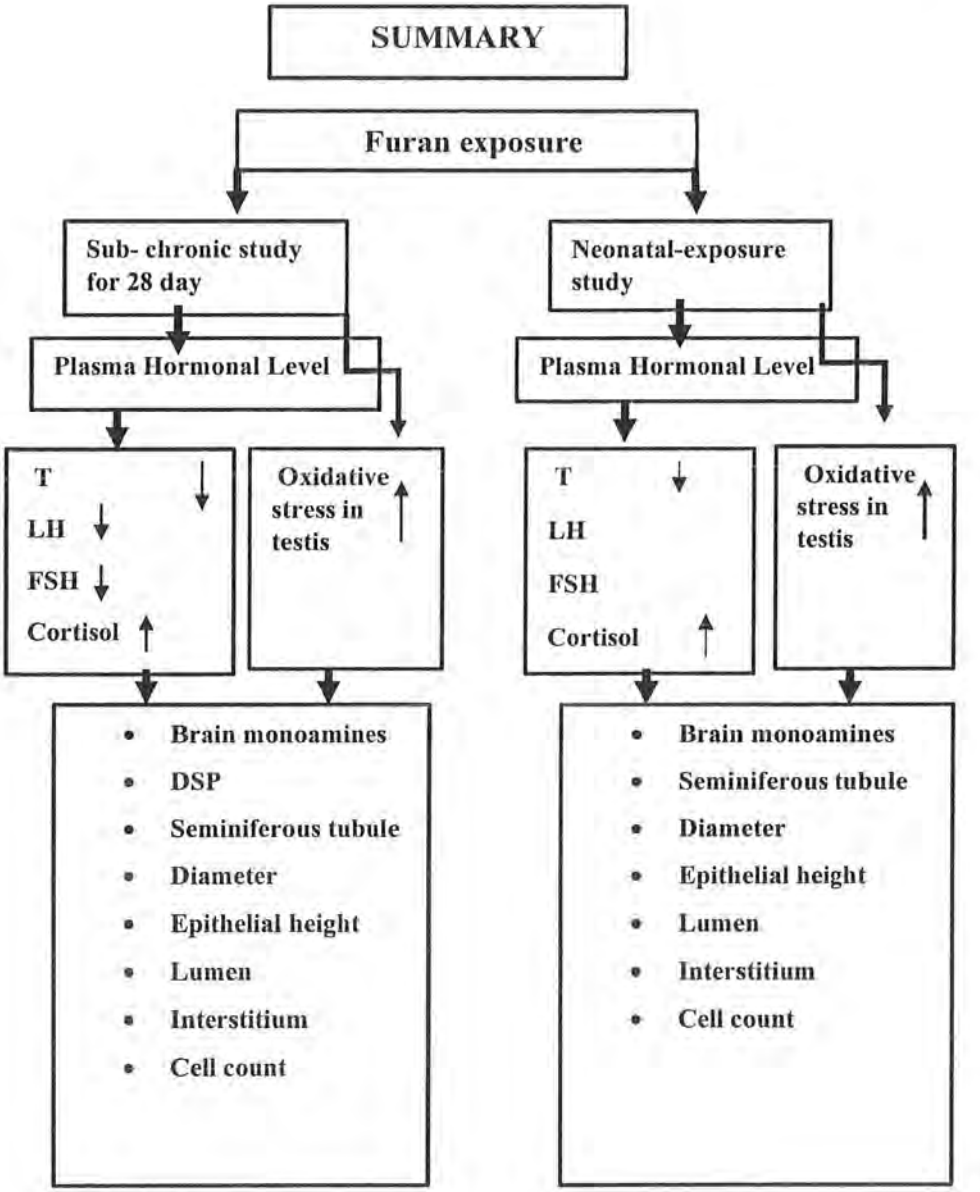


Fig 27. Schematic representation of effect of sub chronic and neonatally *in vivo* furan exposure on rats. Exposure to Furan can suppress gonadotropin secretion, T production and brain monoamines activity increases oxidative stress in the testis and induces histological changes in the testis of rats.

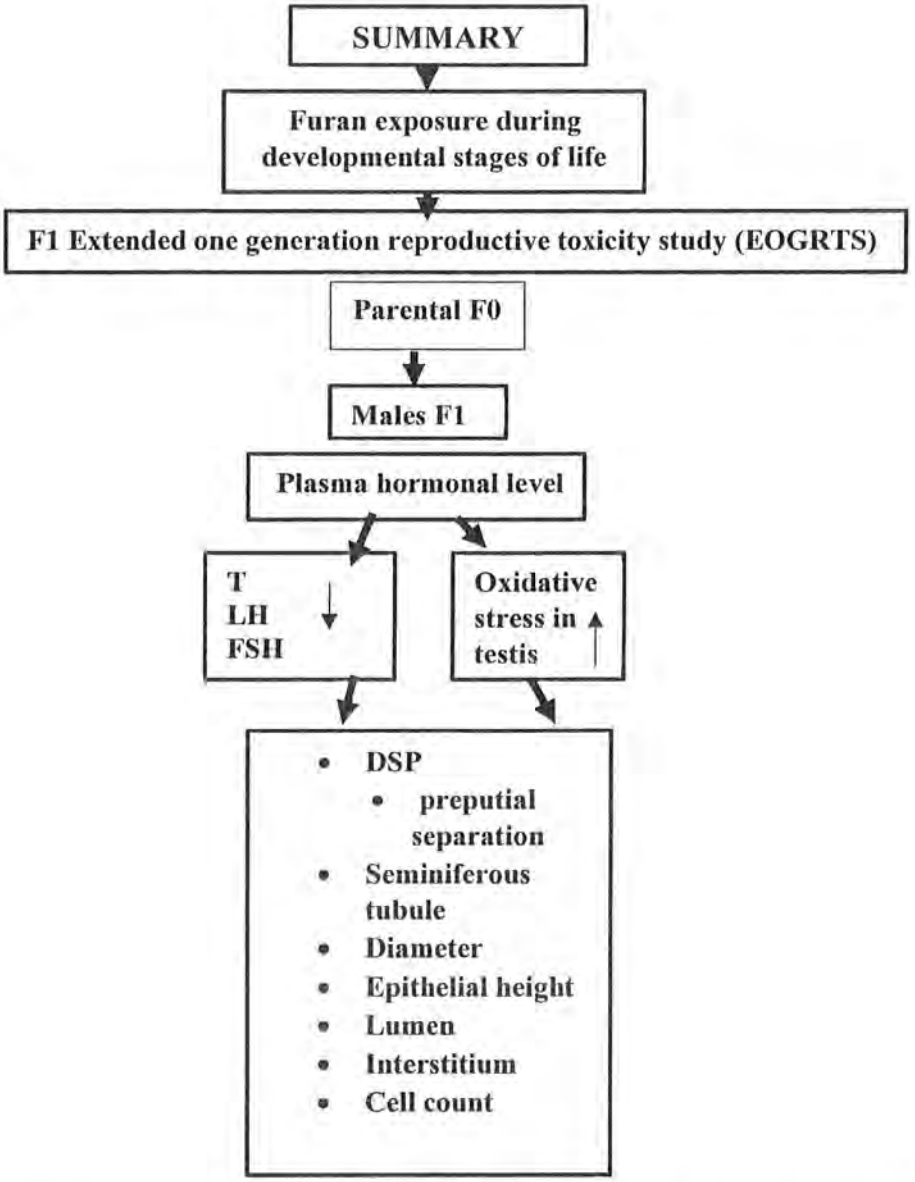
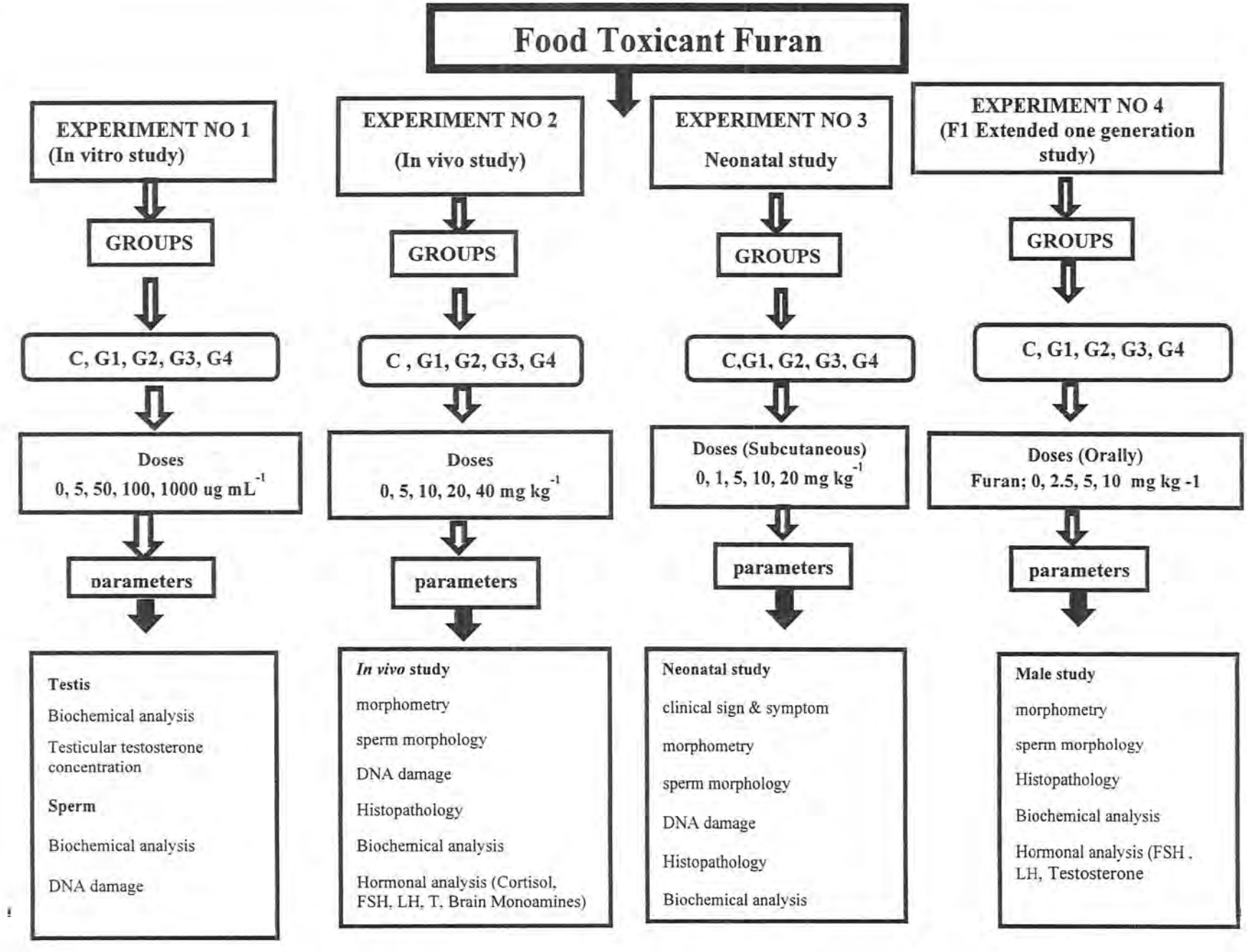


Fig 28. Schematic representation of the effect of furan exposure on F1 Extended one-generation reproductive toxicity study (EOGRTS) rats. Exposure to furan can suppress gonadotropin secretion, T, estrogen production and increases oxidative stress in the testis and ovary, induces histological changes in the testis and ovaries of rats.


Food Toxicant Furan
EXPERIMENT NO 1
(In vitro study)
GROUPS
C, G1, G2, G3, G4
Doses
0, 5, 50, 100, 1000 ug mL-1
parameters
Testis
Biochemical analysis
Testicular testosterone concentration
Sperm
Biochemical analysis
DNA damage
EXPERIMENT NO 2
(In vivo study)
GROUPS
C , G1, G2, G3, G4
Doses
0, 5, 10, 20, 40 mg kg-1
parameters
In vivo study
morphometry
sperm morphology
DNA damage
Histopathology
Biochemical analysis
Hormonal analysis (Cortisol, FSH, LH, T, Brain Monoamines)
EXPERIMENT NO 3
Neonatal study
GROUPS
C,G1, G2, G3, G4
Doses (Subcutaneous)
0, 1, 5, 10, 20 mg kg-1
parameters
Neonatal study
clinical sign & symptom
morphometry
sperm morphology
DNA damage
Histopathology
Biochemical analysis
EXPERIMENT NO 4
(F1 Extended one generation study)
GROUPS
C, G1, G2, G3, G4
Doses (Orally)
Furan; 0, 2.5, 5, 10 mg kg -1
parameters
Male study
morphometry
sperm morphology
Histopathology
Biochemical analysis
Hormonal analysis (FSH , LH, Testosterone

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Toxicological effects of furan on the reproductive system of male rats: An "*in vitro*" and "*in vivo*"-based endocrinological and spermatogonial study

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HIGHLIGHTS

- Different approaches were used to assist furan induced male reproductive toxicity.
- Furan administration induced oxidative stress and toxicity in testicular tissues.
- Furan reduced plasma and testicular Testosterone concentration.

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ABSTRACT

Furan is a colorless toxic chemical produced in various food items during heat processing and in chemical industries. Both *in vitro* and *in vivo* studies have reported that it induces oxidative stress and endocrine disruption; however, limited data are available regarding the effects of furan on the reproduction of mammals. In the present study, an *in vitro* experiment was designed to investigate the direct effects of furan exposure on oxidative stress and testosterone concentration in rat testicular tissue. Furan not only generated high oxidative stress but also decreased antioxidant enzyme activity in the testicular tissue. On the basis of *in vitro* study results, an *in vivo* sub-chronic exposure study was performed. Male rats were orally exposed to different concentrations of furan (0, 5, 10, 20, and 40 mg kg^{-1}). An increase ($P < 0.05$) of reactive oxygen species (ROS) and of the lipid profile (cholesterol, triglycerides, and LDL) in higher dose treatment groups of furan was observed, while total protein content and antioxidant enzyme activity were considerably decreased after furan exposure. Also, plasma and intratesticular testosterone concentrations were reduced in high-dose treatment groups. Sperm parameters such as sperm viability, sperm count, and sperm motility showed a decrease ($P < 0.05$) in a dose-dependent manner. Histopathological findings revealed significant alterations in testis and epididymis tissues. These results confirm that furan can induce toxic effects on the reproductive system of male rats.

1. Introduction

Furan is a colorless heterocyclic organic compound produced in various chemical industries and also formed in a variety of food items during heating processes, such as coffee, sauces, soups, canned, jarred foods, infant formulas, and baby foods (Bolger, 2009; EFSA, 2009; WHO, 2009; Bakhiya and Appel, 2010; Conti et al., 2014a,b; Cooke et al., 2014; FDA, 2004). Furan is also present in the environment as a main constituent of cigarette smoke, wood smoke, and exhaust gases from engines. Furan needs to be removed from consumer products because of its negative effects on human health. Previous observations reported that furan caused various types of cancer and has an adverse effect on the biological system of humans and animals (IARC, 1995; Hamadeh et al., 2004; Pandir, 2015; Uçar and Pandir, 2017). This compound induces liver and hepatic necrosis and DNA damage, which is in turn associated with an increase in serum alanine aminotransferase (ALT) in rats (Gill

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et al., 2010; McDaniel et al., 2012). The metabolite of furan promotes the induction of cell proliferation and disconnection of mitochondrial oxidative phosphorylation (Kedderis, 1999). Bas and Pandir (2016) reported that furan exposure increases malondialdehyde (MDA) levels and decreases glutathione-S-transferase (GST), catalase, and superoxide dismutase in rats.

Thus far, limited information is available in the literature about the toxic effects of furan on the male reproductive system. Furan caused significant changes in histological structure, DNA structure of ovarian cells, malondialdehyde levels, and antioxidant enzyme activities (Uçar and Pandir, 2017). An *in vitro* study showed that furan induced chromosomal aberrations and sister chromatid exchanges (SCEs) in Chinese hamster ovary (CHO) cells and Chinese hamster V79-derived cell line (NTP, 1993; Glatt et al., 2005). Some studies showed antiandrogenic activity of furan (Karacaoglu and Selmanoglu, 2010; Cook et al., 2014). In a human study, furan exposure generated reactive oxygen species and affected the lymphocytes and sperm cells (Pandir, 2015).

In view of these studies, it is concluded that humans are exposed to furan through many ways either directly or indirectly. Hence, there is a need to perform furan risk assessment on the reproductive toxicity of mammals using an appropriate approach. Moreover, limited information is available about the potential adverse effects of furan and other carcinogenic compounds on the reproductive systems of animals (EFSA, 2009). However, because of the complex physiology and regulatory role of the male reproductive system, there is high risk that exposure to toxicants induces many serious toxicological disorders at various sites of the reproductive tissues and organs (Creasy and Foster, 2002). Human exposure to furan has been reported previously, and exposure to this compound increases each day because of its formation in high amounts in home overcooked food and baby products. Furan was also reported to occur in morning urine samples of humans in different concentrations (Ghosh et al., 2015). Therefore, the present investigation was undertaken to elucidate the possible comparative *in vitro* and *in vivo* effects of furan exposure on the mammalian reproductive system by using rats as an animal model.

2. Materials and methods

2.1. Chemicals and animals

Furan (Cat#185922, 99% purity) was purchased from Sigma-Aldrich, 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, 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 rats (age 80–90 days) were collected from the Animal House Facility Quaid-I-Azam University, Islamabad, Pakistan. The animals were housed as a group of 7 rats per cage made of steel. Before the start of the experiment, standard colony conditions were maintained according to Ullah et al. (2016). Handling of animals and experimentation procedures were approved by the Ethical Committee of Animal Sciences Department.

2.2. Experimental design

The experiments were designed to study the effects of furan on the reproductive system of male rats. The *in vitro* experiment was performed to assess the direct effect of furan on testosterone concentration and antioxidant enzymes in the testicular tissues, while the *in vivo* experiment was performed as a sub-chronic exposure study for 28 days to assess the effects of furan on the reproductive system of male rats. Experimental animals were observed to notice any morphological and physiological variations during the experimental time period.

2.3. In vitro experimental design

For the *in vitro* experiment, seven adult male rats were used. This procedure was performed to examine the effect of the direct exposure of furan on testosterone production and testicular tissue antioxidant enzyme status. Different concentrations of furan, i.e., 0, 1, 100, 500, and 1000 $\mu g\ mL^{-1}$, were used in this study. Dose selection was done according to studies on furan exposure to Chinese hamster ovary cells (NTP, 1993). Furan concentrations dissolved in dimethyl sulfoxide (DMSO) and further diluted with cell culture media. The final concentration of DMSO in the media was <1%.The testicular tissues were cultured *in vitro* according to previous protocols (Ullah et al., 2016; Freyberger et al., 2010; Moundipa et al., 2006) with slight modifications. Male rats were euthanized by decapitation. The testes of each rat were cut into five equal parts and placed in culture tubes. The culture medium included 2 mL of Dulbecco's modified Eagle's medium/Ham F12 (DMEM/Ham F12 mixture medium) containing 1.2 $g\ L^{-1}$ sodium bicarbonate and supplemented with 50 IU mL^{-1} penicillin and 50 $\mu g\ mL^{-1}$ streptomycin. Furan solution, at concentrations of 0, 1, 100, 500, and 1000 $\mu g\ mL^{-1}$, was added into the culture tubes, and the tubes were placed in a CO_2 incubator at 33 °C. After incubation for 2 h, the tissues were removed from the CO_2 incubator and washed with cold physiological saline. The tissues (90 mg) were homogenized in 3 mL of phosphate-buffered saline (PBS) and centrifuged at 30,000 rpm for 30 min. The supernatant was stored at −80 °C until it was used for hormonal analysis and antioxidant enzyme assays.

2.4. In vivo sub-chronic exposure experiment

Adult male rats (n = 35) aged 80–90 days old were selected for this experimental procedure. The animals were distributed into 5 groups (n = 7 rats in each group). A stock solution of furan was prepared in corn oil. Furan was orally administered at different concentrations (0, 5, 10, 20, and 40 mg kg^{-1} bw d^{-1}) for 28 days. Dose time was chosen according to the standard protocol 407 of the OECD (2008). Different doses of furan were selected to assess the dose-dependent sub-chronic exposure response. Furan doses used in this experiment were selected on the basis of doses used in previous studies on furan and acrylamide with some slight modifications in concentration (Rawi et al., 2012; Karacaoglu et al., 2010; El-Akabawy et al., 2016; Kara et al., 2016; Dortaj et al., 2017) and OECD guideline # 408 (doses can be used according to the relevant substance if doses have not been previously reported). On day 29, all the selected rats were decapitated; blood samples were collected from the trunk region with pre heparinized syringes, and reproductive organs (testis, epididymis, seminal vesicle and prostrate) were dissected out and weighed using, weigh balance (AND GF-300, JAPAN) and stored for further analysis.

2.5. Blood and tissue collection

The collected blood samples were centrifuged at 3000 rpm for 10 min. After centrifugation, the plasma was collected and frozen at −20 °C for hormonal analysis. After dissection, the tissues (left testis and left epididymis) were properly washed with cold physiological saline and stored in liquid nitrogen for further analysis. The right testis and epididymis tissues were fixed in 10% formalin solution for histological observation.

2.6. Antioxidant enzyme assay

The tissues were collected from both *in vitro* and *in vivo* experiments and processed for antioxidant enzyme analysis. The collected tissues from both experimentations were homogenized in PBS and centrifuged for at least 30 min at 30,000 rpm. The supernatant was collected for protein estimation, antioxidant enzyme assay, and hormonal analysis.

Antioxidant status of the testicular tissue was determined using the supernatant collected from both *in vitro* and *in vivo* experiments. Activity of the plasma antioxidant enzymes catalase (CAT; Aebi, 1984), superoxide dismutase (SOD; Kakkar et al., 1984), and peroxidase (POD; (Carlsberg, 1975); lipid peroxidation (TBARS; Iqbal et al., 1996); and reactive oxygen species (Hayashi et al., 2007) were assessed.

2.7. Total protein

The total protein contents in the tissues were determined using a protein kit purchased from AMEDA Labordiagnostik GmbH, Krenngasse, Graz, Austria (Cat # BR5202-S). The total protein contents were estimated by plotting the absorbance of kit standards versus the absorbance of samples. The total protein contents were expressed in mg g^{-1} of tissue.

2.8. Analysis of serum biochemistry

Blood plasma obtained from the *in vivo* experiment was analyzed for total cholesterol (Cat # REF2230650), triglycerides (Cat # REF4730650), HDL (Cat # BR3251), and LDL (Cat # BR3302) using AMP diagnostic kits (AMEDA Labordiagnostik GmbH, Austria) using a chemistry analyzer according to the manufacturer's instruction manual.

2.9. Sperm parameters

2.9.1. Sperm motility

For "*in vivo*" sperm motility assessment, the cauda of the epididymis was cut and kept in 0.5 mL of pre-warmed PBS (pH 7.3) containing a drop of stain (nigrosin). Fifty microliters of the homogenate was placed on a pre-warmed (at 37 °C) slide for microscopic observation at 40×. At least 10 fields were observed, and 100 sperm per sample were analyzed for motility. The samples were analyzed in triplicate, and the average value was used as the total sperm motility (Halvaei et al., 2012).

2.9.2. Sperm count

The epididymal sperm count was determined in the tissues collected from the *in vivo* experiment. The caudal region of the epididymis was taken in 1 mL of physiological saline solution. The tissues were minced with scissors gently and kept at 37 °C for 15 min. Nigrosin (2–3 drops) was added to the tissues. Using a pipette, 10 μL of the homogenate was placed on a pre-warmed slide. At least 10 fields were observed for spermatozoa count using a microscope at 40×.

2.9.3. Assessment of sperm viability

Eosin-nigrosin staining test was performed to assess sperm viability in tissues obtained from the *in vivo* experiment. The eosin-nigrosin dye (25 μL) was mixed with a semen sample. A drop (15 μL) of this mixture was placed on a glass slide, and a smear was prepared and dried at room temperature. The prepared slides were observed under a microscope at 40×. The live spermatozoa remained white (unstained), whereas the dead spermatozoa were stained red. The percentage of live and dead spermatozoa was calculated by counting at least 100 sperm (Halvaei et al., 2012).

2.10. Hormonal analysis

Intratesticular testosterone and plasma testosterone concentrations were quantified using ELISA kits (Cat # BC-1115, BioCheck Inc., USA) according to standard protocol instructions and information provided by the manufacturer.

2.11. Tissue histopathology

Testis and epididymis collected from the *in vivo* experiment were retained in 10% formalin for 48 h. After fixation, paramount slides of tissues were prepared according to standard protocols as previously described by (Jahan et al., 2016; Ullah et al., 2016). The prepared slides were observed under Leica Microscope equipped with a digital camera (Canon, Japan). Images of all the slides were taken at 40× magnification. Histomorphometric analysis was performed using the Image J2 × software package program.

The areas of seminiferous tubule and epididymis tubules were determined by planimetry using ImageJ software. The calculated area was expressed in square micrometers according to the method of Islam et al. (2009) and anatomical record insight (2013). Shortly, 50 pictures of known area taken at 40× per animal were selected, and the area of the seminiferous tubules was determined using the free selection tool of software. The area in % was calculated using the formula

$$\%As = As * 100/T$$

where As is the area covered by seminiferous tubules, T is the total area of the field.

The percentage of the mean area was calculated for a comparison between the control and treatment groups.

2.12. Statistical analysis

The overall results were expressed as mean ± SEM. All statistical analyses were carried out using easyanova (Arnhold, 2013) and lme4 (Bates et al., 2014) package of R 3.2.5 (R Development Core Team, 2016). The assumption of normality, homogeneity of variances, and additivity of the statistical model were checked using the *Shapiro-Wilk, Levene's,* and *Tukey1-dF test,* respectively. The effects of treatment with different furan concentrations were analyzed using the ANOVA ea1 test command of R program with completely randomized design followed by post hoc *Tukey's HSD.* Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Results of in vitro testicular tissue incubation with different furan concentrations

The testicular tissues were treated with different concentrations of furan for 2 h of incubation with CO_2. After that, levels of the antioxidant enzymes CAT, POD, and SOD; ROS; and LPO were measured. The activities of SOD, CAT, and POD in rat testicular tissue were decreased compared with those of the control group, and the results are shown in Table 1. CAT activity significantly increased in a dose-dependent manner in the treatment groups, which was observed in the 500-μg mL^{-1} ($p = 0.004$) and 1000 μg mL^{-1} ($p = 0.001$) dose groups (Table 1). SOD level did not show statistical difference in any treatment group compared to that of the control group (Table 1). Significantly ($p < 0.001$) decreased levels of POD were also observed in the 500-μg mL^{-1} and

Table 1
Mean (±SEM) result of the *in vitro* effect of furan on testicular antioxidant enzyme and testosterone level in rat testis.

Parameters	Furan Treatments					Statistics
	Control	1 μg mL^{-1}	100 μg mL^{-1}	500 μg mL^{-1}	1000 μg mL^{-1}	P and F values
CAT (U mg^{-1} protein)	22.72 ± 2.58^{a}	18.33 ± 2.63^{ab}	15.07 ± 0.71^{ab}	12.97 ± 0.57^{b}	11.86 ± 0.29^{b}	P < 0.001, F = 6.32
SOD (U mg^{-1} protein)	18.82 ± 4.40^{a}	16.17 ± 2.12^{a}	16.31 ± 0.97^{a}	13.71 ± 1.13^{a}	10.32 ± 1.6^{a}	P = 0.1, F = 1.77
POD (nmol)	4.82 ± 1.53^{a}	4.08 ± 0.44^{c}	3.74 ± 0.79^{c}	1.95 ± 2.7^{b}	1.02 ± 1.5^{c}	P < 0.001, F = 128.36
LPO (nM TBAR min^{-1} mg^{-1})	26.57 ± 2.58^{a}	44.85 ± 7.79^{a}	45.14 ± 8.62^{a}	53.42 ± 8.17^{a}	51 ± 6.24^{a}	P = 0.08, F = 2.22
ROS (U g^{-1} tissue)	35.85 ± 3.28^{a}	38.57 ± 1.21^{a}	40.14 ± 2.62^{a}	56 ± 1.67^{a}	59.57 ± 2.27^{a}	P = 0.1, F = 1.29
Testosterone (ngg^{-1} tissue)	68.14 ± 2.43^{a}	53.28 ± 1.97^{ab}	47 ± 2.90^{ab}	49.71 ± 2.90^{ab}	25.71 ± 1.34^{b}	P = 0.06,F = 2.48

P and F values in the rows were obtained from ANOVA with completely randomized simple designs, followed by Tukey's post hoc analysis, which shows a pairwise comparison of control and furan-treated groups. Mean values with different superscripts show significant difference ($p < 0.05$) in the columns when comparing control with other treatment groups.

1000 μg mL^{-1} dose groups when compared with those of the control group. A substantial difference was observed in ROS and LPO levels ($P = 0.08$) as compared to those of the control group (Table 1).

Testosterone concentration (68.14 ± 2.43, 53.28 ± 1.97, 47 ± 2.90, 49.71 ± 2.90, and 25.71 ± 1.34) was observed after 2 h of incubation with various doses of furan. Testosterone concentration was significantly ($p = 0.03$) decreased in the 1000 μg mL^{-1} dose group when compared with that of the control group. Testosterone concentration also decreased in other treatment groups, but the decrease was not statistically significant (Table 1).

3.2. Results of effects of in vivo orally administered furan on the weight of rat's body, testis and epididymis, and accessory organs

There was no significant change in animal weight gain among all groups ($p = 0.95$) (Table 2). Furan treatment did not alter the weights of testis, epididymis, seminal vesicles, and prostate gland (Table 2).

3.3. Results of effects of in vivo orally administered furan on biochemical parameters of rat testis

Data of total protein content, ROS, and antioxidant enzyme activity of the control and treatment groups are presented in Table 3. After 28 days of the furan treatment, values of SOD and POD were significantly ($P < 0.05$) decreased in higher dose treatment groups than in the control group. A decrease in CAT activity was also seen in higher dose treatment groups. However, a minor increase in LPO activity was observed in the high-dose treatment groups, but this increase was not statistically significant (Table 3). Activity of ROS was significantly ($P < 0.05$) increased in higher dose groups, i.e., 20 mg kg^{-1} and 40 mg kg^{-1} treatment groups. A significant ($P = 0.001$) decrease in protein content was observed in high-dose treatment groups, i.e., 10 mg kg^{-1}, 20 mg kg^{-1}, and 40 mg kg^{-1} after 28 days of furan exposure (Table 3). Blood plasma levels of triglyceride, cholesterol, LDL, and HDL for all treatment groups are presented in Table 3. A significant increase ($p = 0.001$) in triglyceride, cholesterol, and LDL levels but a decrease in HDL ($p < 0.001$) was estimated in high-dose groups (10 mg kg^{-1}, 20 mg kg^{-1}, and 40 mg kg^{-1}) versus the control group (Table 3).

3.4. Results of effects of oral administration of furan on sperm parameters

Furan caused a significant decrease in sperm motility ($P = 0.007$), sperm count ($p < 0.001$), and sperm viability ($p < 0.001$) in the high-dose furan-treated groups when compared with the control group (Table 3).

3.5. Results of the effects of the orally administered furan on plasma and intratesticular testosterone in rat testis

Intratesticular and plasma testosterone concentrations in control and furan-treated groups are shown in Table 3. Plasma testosterone concentration was significantly ($p = 0.04$) lowered in the high-dose (40 mg kg^{-1}) treatment group. Similarly, a significant ($p = 0.02$) reduction in intratesticular testosterone concentration

Table 2
Mean (±SEM) results of body weight and relative organ weight of rats in the control and furan-treated groups.

Parameters	Furan (mg kg^{-1} d^{-1})					
	Control	5 mg kg^{-1}	10 mg kg^{-1}	20 mg kg^{-1}	40 mg kg^{-1}	P and F value
Body weight gain (g)	54.31 ± 2.15	50 ± 4.59	51.6 ± 2.71	51.6 ± 4.11	51.2 ± 4.84	P = 0.95 F = 0.16
Right testis weight (g)	1.37 ± 0.04	1.31 ± 0.10	1.24 ± 0.04	1.33 ± 0.08	1.32 ± 0.09	P = 0.83 F = 0.35
Left testis weight (g)	1.30 ± 0.11	1.29 ± 0.14	1.21 ± 0.09	1.13 ± 0.08	1.12 ± 0.09	P = 0.63 F = 0.64
Right epididymis weight (g)	0.68 ± 0.05	0.60 ± 0.09	0.62 ± 0.05	0.69 ± 0.05	0.75 ± 0.07	P = 0.46 F = 0.93
Left epididymis weight (g)	0.63 ± 0.07	0.45 ± 0.08	0.59 ± 0.02	0.73 ± 0.04	0.69 ± 0.10	P = 0.11 F = 2.13
Prostate (g)	0.63 ± 0.05	0.57 ± 0.09	0.44 ± 0.07	0.47 ± 0.08	0.51 ± 0.09	P = 0.47 F = 0.91
Seminal vesicle (g)	0.73 ± 0.24	0.59 ± 0.08	0.51 ± 0.04	0.38 ± 0.02	0.38 ± 0.04	P = 0.21 F = 1.59

P and F values in the rows were obtained from ANOVA with completely randomized simple designs, followed by Tukey's post hoc analysis, which shows a pairwise comparison of control and furan-treated groups. Mean values with different superscripts show significant difference ($p < 0.05$) in the columns when comparing control with other treatment groups.

Table 3
Mean (±SEM) values of *in vivo* effect of furan on biochemical parameters, sperm parameters, and hormonal concentration of rat testis treated with different doses of furan.

Parameters	Furan (mg kg^{-1} d^{-1})					Statistics
	Control	5 mg kg^{-1}	10 mg kg^{-1}	20 mg kg^{-1}	40 mg kg^{-1}	P and F values
SOD (U mg^{-1} protein)	23.35 ± 2.15^{a}	20.09 ± 2.36^{ab}	20.48 ± 1.48^{a}	15.58 ± 1.60^{ab}	12.16 ± 1.64^{b}	P = 0.003, F = 5.57
POD (nmol min^{-1} mg^{-1} protein)	15.73 ± 1.79^{a}	14.40 ± 2.97^{ab}	9.14 ± 1.52^{bc}	7.86 ± 1.01^{c}	11.46 ± 1.87^{ac}	P = 0.04, F = 2.96
LPO (nM TBAR min^{-1} mg^{-1})	1.81 ± 0.35^{a}	1.94 ± 0.41^{a}	2.04 ± 0.44^{a}	7.65 ± 1.06^{a}	6.34 ± 1.81^{a}	P = 0.54, F = 0.79
Total ROS (U mg^{-1} tissue)	1.38 ± 0.07^{b}	2.02 ± 0.09^{b}	2.41 ± 0.04^{ab}	3.07 ± 0.15^{ab}	3.33 ± 0.06^{a}	P = 0.05, F = 2.86
Protein (mg $0.5\ g^{-1}$)	60.78 ± 2.66^{a}	49.32 ± 1.89^{b}	43.23 ± 2.53^{bc}	42.65 ± 2.34^{bc}	36.28 ± 2.85^{c}	P = 0.001, F = 13.8
Cholesterol (mg dl^{-1})	46.2 ± 2.58^{d}	48 ± 1.59^{cd}	64 ± 2.22^{b}	58.4 ± 2.02^{bc}	72.2 ± 1.77^{a}	P = 0.001, F = 29.8
Triglyceride (mg dl^{-1})	76.71 ± 1.60^{e}	93.2 ± 2.58^{d}	104.8 ± 1.74^{c}	120.8 ± 2.46^{b}	136 ± 0.95^{a}	P = 0.001, F = 139.51
HDL (mg dl^{-1})	60.16 ± 1.94^{a}	40.51 ± 2.78^{b}	$37.2 \pm 3.34b^{b}$	35.7 ± 2.29^{b}	34 ± 0.70^{b}	P < 0.001, F = 19.97
LDL (mg dl^{-1})	34.6 ± 3.86^{c}	51.8 ± 2.99^{b}	51.2 ± 2.22^{b}	59.2 ± 1.68^{b}	70.8 ± 1.32^{a}	P = 0.001, F = 26.15
Sperm count ($\times 10^{6}$/gm of cauda)	176 ± 5.09^{a}	158 ± 3.11^{ab}	150.6 ± 3.59^{bc}	129.3 ± 5.80^{cd}	123.3 ± 6.23^{d}	P = 0.001, F = 19.78
Sperm viability %	84.33 ± 3.82^{a}	74.66 ± 2.65^{ab}	72.33 ± 2.71^{ab}	60.66 ± 2.37^{b}	65.33 ± 3.82^{b}	P = 0.002, F = 8.69
Sperm motility%	67.66 ± 2.37^{a}	56.66 ± 4.49^{ab}	55 ± 5.88^{ab}	46 ± 6.79^{b}	43.6 ± 1.89^{b}	P = 0.02, F = 4.70
Plasma Testosterone (ng ml^{-1})	4.56 ± 0.46^{a}	3.47 ± 0.02^{ab}	3.09 ± 0.46^{ab}	3.39 ± 0.19^{ab}	2.18 ± 0.26^{b}	P = 0.05, F = 3.40
Intra-testicular Testosterone (ng g^{-1} tissue)	26.27 ± 2.82^{a}	22.83 ± 2.54^{ab}	18.61 ± 2.96^{bc}	16.88 ± 1.37^{bc}	14.61 ± 1.30^{c}	P = 0.02, F = 7.57

P and F values in the rows were obtained from ANOVA with completely randomized designs, followed by Tukey's post hoc analysis, which shows a pairwise comparison of control and furan-treated groups. Mean values with different superscripts show significant difference ($p < 0.05$) in the rows when comparing control with other treatment groups.

was also seen in the treatment group when compared with that in the control group, while in the other treatment groups, no significant differences were observed.

3.6. Histopathological results

3.6.1. Testis

Different parameters of histopathology findings of the testicular and epididymal tissues are mentioned in Table 4 and Fig. 1. Testicular tubular and testicular lumen diameters showed significant ($p < 0.001$) difference when compared with the control group. High-dose (20 mg kg^{-1} and 40 mg kg^{-1}) groups showed significant ($p = 0.001$) decrease in tubular diameter and significant ($p = 0.001$) increase in lumen diameter of the testis. However, epithelial height was found to be significantly ($p = 0.05$) reduced in the high-dose treatment groups as compared to that in the control group. A significant ($p = 0.05$) decrease was observed in tunica albuginea height in the 40 mg kg^{-1} treatment group when compared with that in the control group.

3.6.2. Caput epididymis

Histopathology of the epididymal caput region showed no visible changes in caput epithelial heights and lumen diameters (Fig. 2). However, caput tubular diameter showed significant ($p < 0.001$) difference in the high-dose (20 mg kg^{-1} and 40 mg kg^{-1}) groups when compared with that in the control group. Epithelium% and lumen% also showed some differences between the control and treatment groups, but these differences were not highly significant (Table 4, Fig. 2).

3.6.3. Cauda epididymis

The ductular and lumen diameter of cauda showed nonsignificant ($p = 0.93$) changes. Epithelial height of the cauda epididymis was significantly ($p = 0.005$) reduced in the high-dose groups when compared with that in the control group. Epithelium percentage and lumen percentage also showed some differences between control and treatment groups, but this difference was not significant (Table 4, Fig. 3).

4. Discussion

Furan is formed from several precursor molecules naturally present in food during various steps of processing, which might involve oxidation or Maillard reaction (Limacher et al., 2007; Limacher et al., 2007; Bolger, 2009). It has been previously reported that furan is a toxic agent that causes various harmful effects on

Table 4
(Mean ± SEM) values of histopathology of rat testis, epididymis caput, and caudal regions treated with different doses of furan.

Parameters	Furan (mg kg-1 d-1)					Statistics
	Control	5 mg kg-1	10 mg kg-1	20 mg kg-1	40 mg kg-1	P and F Values
Testis	Seminiferous tubule diameter (μm)	238.46 ± 6.82a	236.6 ± 6.91a	212.31 ± 6.48b	178.95 ± 7.70c	163.34 ± 5.79c	P = 0.001, F = 24.99
	Tubular lumen diameter (μm)	26.65 ± 1.14c	27.91 ± 0.75c	32.39 ± 0.74b	37.57 ± 0.83a	36.09 ± 1.10 ab	P = 0.001, F = 24.9
	Epithelial height (μm)	92.67 ± 5.42a	89.47 ± 4.33a	88.68 ± 4.49 ab	81.82 ± 4.51 ab	75.95 ± 2.62b	P = 0.05, F = 2.34
	Tunica albugenia height (μm)	23.92 ± 1.53a	20.79 ± 1.39 ab	20.65 ± 1.11 ab	18.10 ± 1.34 ab	16.09 ± 0.84b	P = 0.04, F = 2.63
Epididymis caput region	Tubule diameter (μm)	333.07 ± 8.26a	317.46 ± 7.83 ab	306.30 ± 7.51b	272.85 ± 6.23c	250.35 ± 6.94d	P = 0.001, F = 20.0
	Lumen diameter (μm)	240.84 ± 7.65a	247.66 ± 9.69a	257.02 ± 7.30a	257.02 ± 7.30a	257.02 ± 7.30a	P = 0.50, F = 0.83
	Epithelial height (μm)	32.89 ± 1.143a	29.77 ± 1.43ac	30.84 ± 1.48 ab	27.76 ± 1.43bc	26.20 ± 1.56c	P = 0.01, F = 3.25
	Epithelium %	41.12 ± 1.42 a	39.12 ± 1.67 a	38.55 ± 1.85 a	36.73 ± 1.90 a	35.171 ± 1.85 a	P = 0.16, F = 1.63
	Lumen%	58.87 ± 1.42b	62.77 ± 1.79 ab	61.44 ± 1.85 ab	65.29 ± 1.79 ab	67.23 ± 1.95a	P = 0.01, F = 3.259
Epididymis caudal region	Tubule diameter (μm)	462.73 ± 7.64 a	460.34 ± 10.16 a	457.82 ± 8.46 a	454.88 ± 9.05 a	450.73 ± 9.66 a	P = 0.83, F = 0.36
	Lumen diameter (μm)	417.76 ± 10.4 a	415.25 ± 9.2 a	413.34 ± 7.47 a	408.19 ± 6.4 a	410.46 ± 8.75 a	P = 0.93, F = 0.199
	Epithelial height (μm)	35.80 ± 1.15a	34.84 ± 1.42a	29.11 ± 1.5 ab	31.13 ± 1.55b	28.76 ± 1.49b	P = 0.01, F = 5.166
	Epithelium%	44.75 ± 1.44a	43.55 ± 1.77 ab	36.39 ± 1.8ac	39.1 ± 1.94bc	35.96 ± 1.87c	P = 0.001, F = 4.16
	Lumen%	55.24 ± 1.44 a	56.44 ± 1.77 a	61.29 ± 2.02 a	58.55 ± 2.09 a	61.15 ± 2.11 a	P = 0.08, F = 2.082

P and F values in the rows were obtained from ANOVA with completely randomized designs, followed by Tukey's post hoc analysis, which shows a pairwise comparison of control and furan-treated groups. Mean values with different superscripts show significant difference ($p < 0.05$) in the rows when comparing control with the other treatment groups.

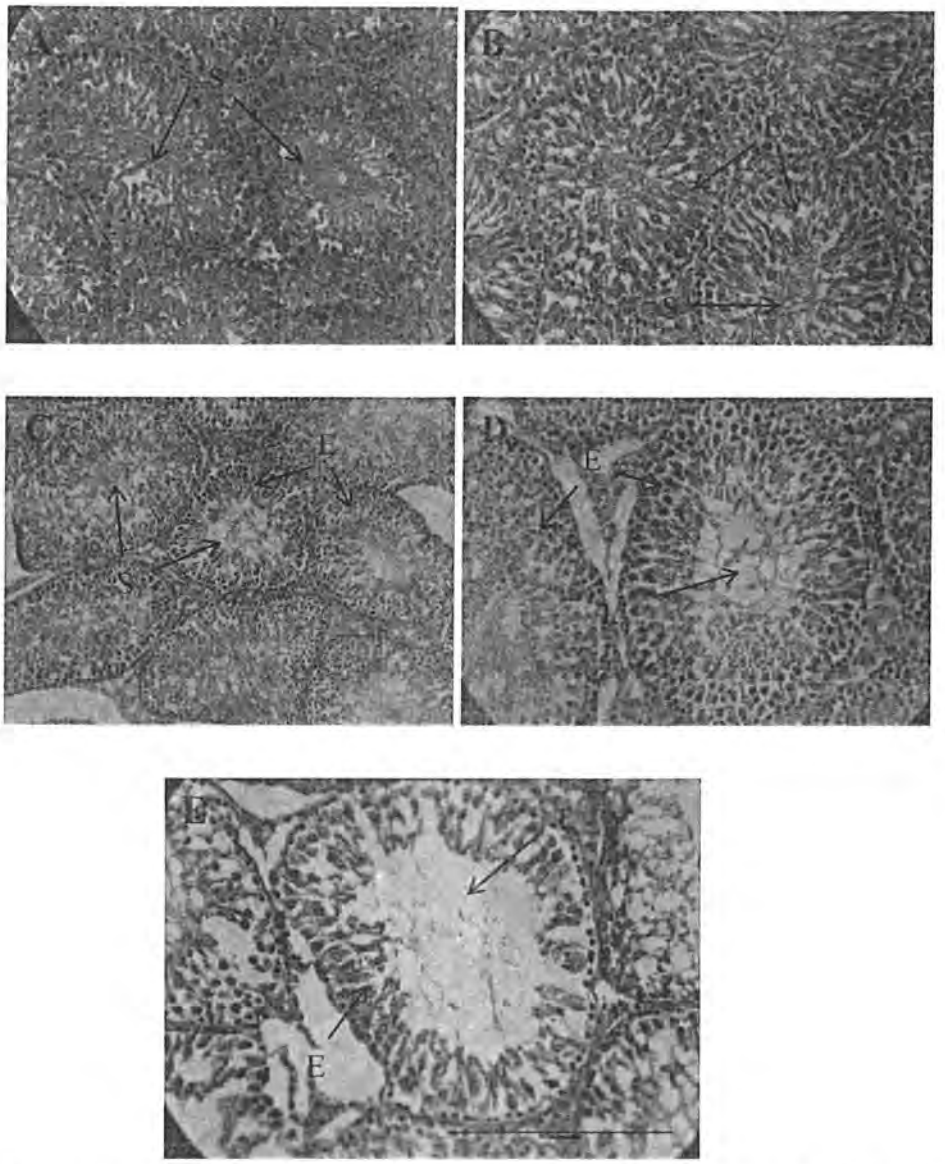

Fig. 1. Photomicrograph of seminiferous tubules of male rats receiving doses of furan. (A) Control; showing regularly arranged tubules, lumen filled with spermatids with normal germ cells (B) 5 mg kg^{-1} group; showing the normal process of spermatogenesis, lumen filled with mature spermatozoa, (C) 10 mg kg^{-1} group; normal morphology with slightly widened lumen (D) 20 mg kg^{-1} group; showing increased lumen diameter and amplified epithelial height, tubules with interstitial spaces are present, (E) 40 mg kg^{-1} group; thin degenerated epithelium with empty lumen. Magnification ×40. Lumen (L), Epithelium (E).

biological systems of living organisms (Pandir, 2015; Uçar and Pandir, 2017; Webster et al., 2014; Gill et al., 2014; Jackson et al., 2014). Previous literature on furan reported that its exposure causes reproductive impairment by disturbing spermatogenesis, thereby leading to apoptosis in germ cell lining and Leydig cells. Thus, on a global scale, furan is of high concern because of its harmful effects on animals and humans (Hamadeh et al., 2004). The present study was designed to investigate the effect of furan on testicular tissue by an *in vitro* experiment and on reproductive functions by an *in vivo* experiment. The results of *in vitro* study showed decreased levels of antioxidants (CAT, SOD, and POD) in testicular tissue. While reactive oxygen species (ROS) and lipid peroxidation (LPO) levels were elevated in testicular tissues by furan treatment as compared to those in the control group. These results are similar to those reported in previous studies in which furan and acrylamide exposure resulted in an elevated level of LPO and reactive oxygen species in the cells (Mehri et al., 2014; Zhao et al., 2013; Pandir, 2015). Similarly, *in vivo* sub-chronic exposure study results indicated that higher doses of furan induced more reproductive toxicity than lower doses. High doses of furan, i.e., 20 and 40 mg kg^{-1}, produce more ROS and oxidative damage in testicular tissues by reducing the seminiferous tubular lumen diameter, epithelial height and decreasing the number of sperm in the lumen of the epididymis. All these changes may be due to high ROS production. oxygen-containing molecules that have free unpaired electrons and are highly reactive. Some nonradical molecules also represent as ROS. These compounds generate free oxygen ions during normal metabolism. Oxidative phosphorylation occurs

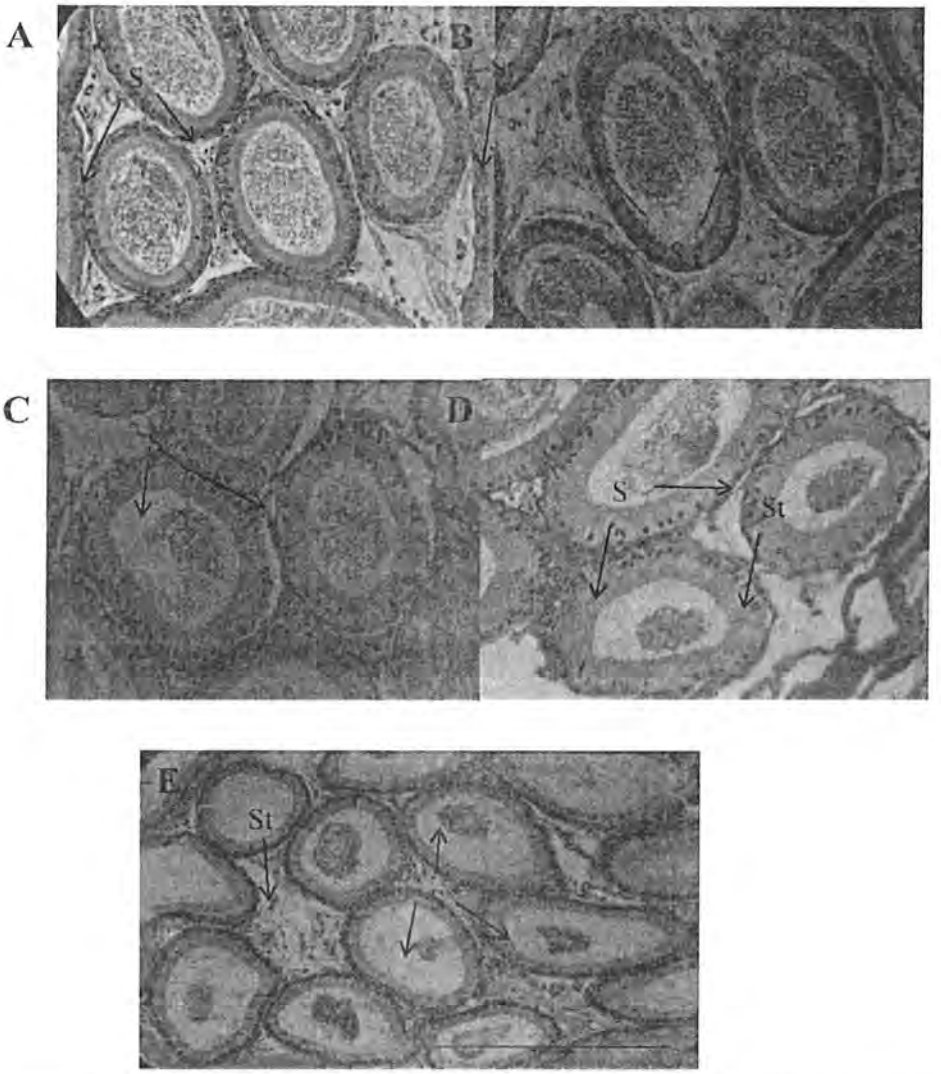

Fig. 2. Photomicrograph of cross-section of caput epididymis (H&E, 40×) of male rats receiving doses of furan showing (A) Control; exhibiting normal morphology of caput epididymis; compactly arranged tubules with thick epithelium, lumen filled with mature sperms, (B) 5 mg kg $^{-1}$ group; showing normal morphology like control. (C) 10 mg kg $^{-1}$ group; showing slight changes in morphology of tubules, surrounded by stroma. (D) 20 mg kg $^{-1}$ group; lumen has very less number of spermatozoa. (E) 40 mg kg $^{-1}$ group; showing a further increase in epithelial height and a slight increase in lumen sperm concentration. Spermatozoa (*S*), Stroma (St).

in mitochondria, which results in the production of ROS (Devasagayam et al., 2004). High ROS production imparts damaging effects on protein, lipids, and DNA (RADak et al., 1999). Self-defense mechanism (antioxidant enzyme) is activated in response to the production of ROS in the cells, which results in the reduction of ROS levels (Kaul and Forman, 2000). The decrease in the antioxidant enzyme levels devastates the cell's sensitivity to harbor oxidative stress, hence compromising its ability to detoxify the effects of ROS (Kaul and Forman, 2000; Pérez et al., 2009). Previously, furan was found to be a cytotoxic, genotoxic, and an apoptotic inducer (Heppner and Schlatter, 2007; Karacaoğlu and Selmanoğlu, 2010). It has already been reported in previous literature that oxidative stress results in the degradation of protein and lipids (RADak et al., 1999). Lipids have important structural and functional roles in different body organs and cells (Rawi et al., 2012) and are also important for maintaining body functions. Oxidative stress causes derangement in serum lipid concentration and disturbance in lipid profile, also called dyslipidemia (abnormal amount of triglycerides, cholesterol, and fat phospholipids) in the bloodstream (Martins et al., 2018). In the current study, plasma total cholesterol, triglyceride, and LDL levels were elevated in furan-treated animals, while the HDL level was reduced. According to previous literature, furan and acrylamide administration causes an increase in plasma LDL, total cholesterol, and triglycerides and decrease in HDL (Ghanayem et al., 2010; Raju et al., 2015). Previously, researchers have reported that association of increased cholesterol and reduced HDL levels is linked to male infertility, low testosterone level, and sperm abnormality (Shalaby et al., 2004).

In the current study, a clear decrease was observed in sperm count. Similar findings were also previously reported by Uzunhisarcikli (2007), where sperm count reduced as a result of reduction in testosterone level. Spermatozoa exposed to high concentration of ROS resulted in reduced sperm viability and motility, leading to infertility (Aitken and Curry, 2011) as

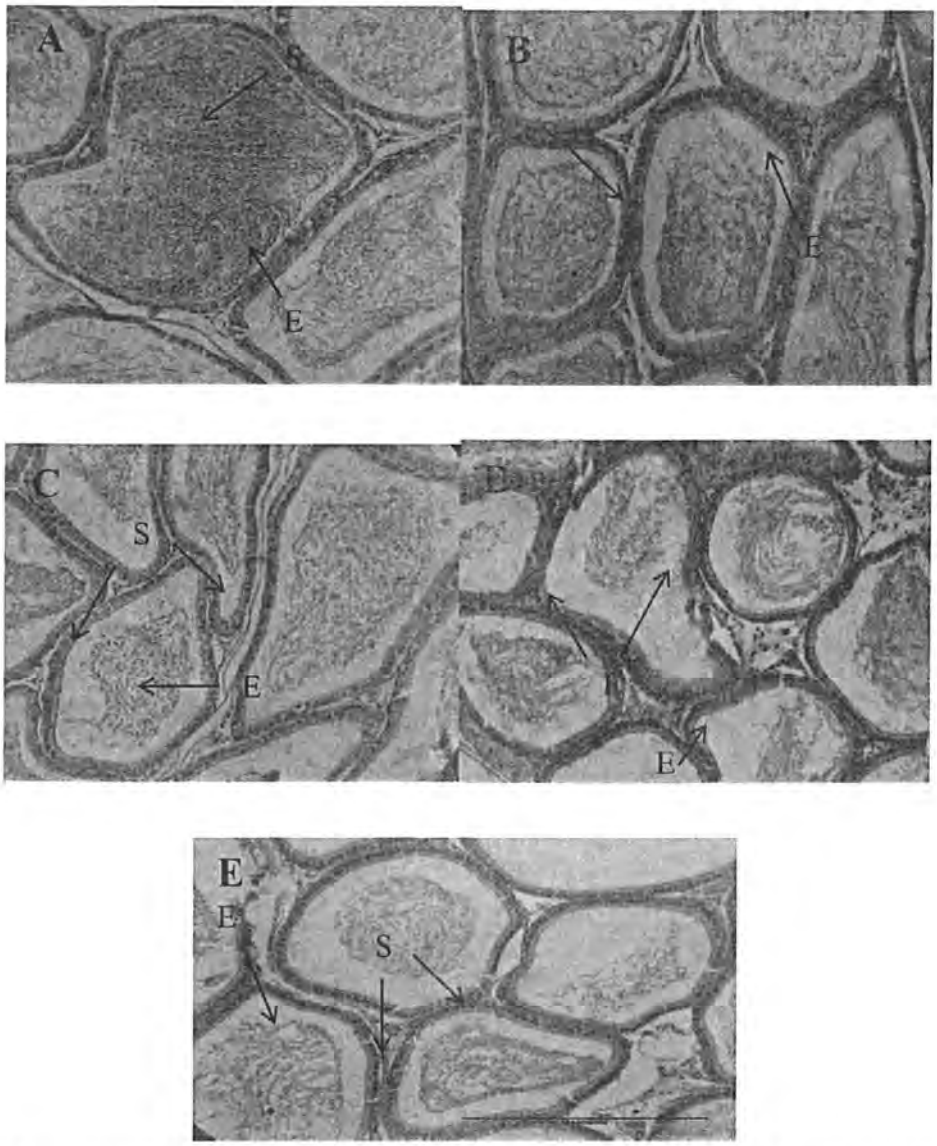

Fig. 3. Photomicrograph of cauda epididymis of adult male rats receiving doses of furan (H&E, 40X) from (A) control group; with normal morphology of cauda epididymal cells having thin pseudostratified epithelium of the lumen heavily filled with spermatozoa, (B) 5 mg kg^{-1} group; with thin epithelium lined with stereocilia, (C) 10 mg kg^{-1} group; showing normal spermatozoa in lumen (D) 20 mg kg^{-1} group; showing increase in pseudostratified epithelium and very little concentration of spermatozoa, (E) 40 mg kg^{-1} group; further reduction in spermatozoa and increased epithelial height. Epithelium (E), Spermatozoa (S).

reproductive hormones control spermatogenesis and cell-to-cell interaction within the testis. In the present study, histopathological findings showed an increase in seminiferous tubular diameter and lumen diameter, while epithelial height declined in a dose-dependent manner. Seminiferous tubules with hollow lumen and less interstitial spaces were observed in high-dose furan administration. These histopathological findings showed the endocrine-disrupting nature of furan. As reported in previous studies, decline in the height of the seminiferous epithelium may be connected to the reduction in testosterone levels, and decreased testosterone levels reduce the number of germ cells in stages VII–VIII of the spermatogenic cycle (Kumar et al., 2006; Karacaoglu and Selmanoglu, 2010).

In the present study, testosterone concentration decreased in groups exposed to high doses of furan. However, a decreased level of testosterone is the indicator of chemical toxicity (Yoshida et al., 2002). As previously described, reduced testosterone concentration is due to oxidative stress, which is connected to the main action of antioxidant enzymes within Leydig cells and enhances the sensitivity of spermatogenesis toward oxidative stress (Cao et al., 2004; Rezvanfar et al., 2013). From the present study, it is concluded that Furan exerts its effects by induction of oxidative stress that also alters testosterone secretion in testis, resulting in decreased sperm production and spermatogenic arrest.

5. Conclusion

Findings of the current study revealed that exposure to high concentration of furan can produce oxidative stress and exhibit antiandrogenic effects that further induce alterations in

reproductive functions of adult male rats. However, we suggest a detailed molecular-based study to know the exact mechanism of action of furan in reproduction.

Declaration of interest

All the authors who have a major contribution in this research article declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2019.05.063.

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Neonatal exposure to furan alters the development of reproductive systems in adult male Sprague Dawley rats

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ABSTRACT

Furan is a colorless toxic organic compound that is produced during thermal degradation of natural food constituents, and is present in various processed foods such as coffee and processed baby foods. The present study investigated the endocrine disrupting potential of furan in Sprague Dawley male pups. On postnatal day 0 (PND 0), pups were divided into five groups. The control group received subcutaneous injections of corn oil (50 μL), while the treated groups were injected with one of four concentrations of furan (1, 5, 10 and 20 mg kg^{-1} d^{-1} in 50 μL corn oil) from PND 1 to PND 10. Our results reveal significant physiological changes in groups receiving the two highest doses of furan (10 and 20 mg kg^{-1} d^{-1}). Fertility was decreased in high dose groups, as evidenced by lower daily sperm production (DSP) and epididymis sperm counts, and dose-dependent histological alterations in the testes. High dose groups showed significant reductions in plasma concentrations of testosterone, LH and GH, while plasma cortisol and final body weight was increased compared to the control group. The results suggest that neonatal exposure to high concentrations of furan cause structural and endocrine alterations in male neonatal rats, compromising fertility.

1. Introduction

The recent decades have seen a rise in public attention for the adverse effects of chemicals on human health. Various environmental chemicals have been classified as endocrine disrupting chemicals (EDCs) because of their ability to alter animal physiology by disrupting hormonal levels. Especially developmental stages are vulnerable, since changes can become permanent when animals are exposed to EDCs during organ development (Richter et al., 2007), changing the hormonal profile irreversibly (Cooke et al., 2014; Hinson and Raven, 2006; Lafuente et al., 2003; Liu et al., 2010; Sajjad et al., 2018). Such irreversible and long-lasting changes are termed "organizational effects" (Arnold and Breedlove, 1985; Young, 1964). On the other hand, EDCs may be less harmfull after gonodal development is completed, as exemplified by the observation that men exposed to polychlorinated biphenyls at the age of 20 do not show a bias in the number of sons they conceive (del Rio Gomez et al., 2002).

In human infants, the main pathway for exposure to environmental toxicants is via the diet, either from breastfeeding or formula feeding (Lehmann et al., 2018). It is well documented that environmental toxicants like bisphenol, arsenic, chlorinated dioxins, TCDD, furan, polychlorinated biphenyls, DDT and acrylamide are present in breast milk and infant formula foods (Lehmann et al., 2018; Mojska et al., 2012). In males, exposure to the dioxin TCDD between 1 and 9 years is associated with a reduction in sperm counts and sperm motility (Mocarelli et al., 2007) along with a decrease in Sertoli cell number in adulthood (Sharpe et al., 2003). In female infants, exposure to polybrominated biphenyls via breastmilk has been linked to early menarche (Blanck et al., 2000). In addition to these more well-known EDCs, infants and children may are exposed to heat-induced food toxicants such as furan and acrylamide, which also have endocrine disrupting potential (FDA, 2004; Robin and Clanet, 2007). Furan (C_4H_4O) belongs to a group of dioxins (polychlorinated dibenzo-furans, PCDFs) that are produced during thermal degradation of natural food constituents. Furan is found in many processed foods including jarred and canned foods, coffee and infant formulas (Karacaoğlu and Selmanoğlu, 2010; Lehmann et al., 2018; Pluim et al., 1993).

The effect of developmental exposure to furan has been evaluated only in a handful of studies, despite strong experimental and epidemiological evidence that early life stages are highly sensitive to dioxins (Berga, 2008; Birnbaum and Tuomisto, 2000; Peterson et al., 1993; van den Berg et al., 2017). In addition to increased sensitivity, infants have

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higher intake rates of furan as compared to adults, because they rely solely on breastmilk or formula milk which contain high concentrations of furan. Exposure may even start in utero, since furan readily passes the placental barrier (Van Wijnen et al., 1990).A clinical study reported that 11-week old babies had higher thyroid hormone levels when their mother's breastmilk contained higher than average dioxin and furan concentrations (Pluim et al., 1993). In adult rats, exposure to furan resulted in decreased testosterone levels, decreased sperm counts, impaired spermatogenesis and induced apoptosis in Leydig and germ cells (Cooke et al., 2014; Karacaoglu and Selmanoğlu, 2010). The present study investigated the effect of neonatal exposure to furan on the reproductive system of male rats. We evaluated sexual maturation, sperm parameters, hormonal profile, and histopathological parameters.

2. Materials and methods

2.1. Animal care and maintenance

Animal treatment and experimental rules were officially permitted by the ethical committee of the Department of Animal Sciences, Quaid-i-Azam University in Islamabad, Pakistan. Sprague-Dawley rats, 8 weeks of age, were obtained from the rodent facility of the same department. Animals were acclimatized to the laboratory for two weeks under the following standard conditions. They were fed with laboratory pelleted chow (Sial Scientific Company, Pakistan) and ad libitum access to tap water. The room temperature was maintained at 22–25 °C, humidity at 50 ± 5%, and the photoperiod was 10 h light and 14 h dark. At 10–11 weeks of age, estrous stage females (n = 30) in breeding cagescontaining a male rat. Vaginal smears were collected on consecutive days until the presence of sperm was confirmed. This day was designated as day 1 of gestation (GD1). Pregnant females were kept in single cages until the birth of pups on gestational day 22 (GD22). The day of birth of the pups was considered as postnatal day 0 (PND0). The number of pups born was recorded, and the sex was determined by measuring the anogenital distance (AGD) using a stereomicroscope. Male and female pups were placed in separate cages.

2.2. Experimental design

On PND0, male pups (n = 50) were distributed into five treatment groups (10 pups per treatment group). The control group received a daily SC injection with corn oil (50 μL) from PND1-10, while treated groups received SC injections with furan (either 1, 5, 10 or 20 mg kg^{-1} d^{-1} in 50 μL corn oil) for ten days. The doses were selected according to previous exposure studies with modifications (Karacaoğlu and Selmanoğlu, 2010; Kim et al., 2004; Rawi et al., 2012). We chose to administer furan using SC injections since neonatal rodents have low liver enzymatic activity, therefore there is no difference between oral and non-oral administration (Chapin et al., 2008; Taylor et al., 2008). Animals were kept in these treatment groups for three months. Their developmental stage was monitored to study the following parameters.

2.3. Determination of body weight

Animals were weighed on PND30, PND45, PND80 and on day of preputial separation (see below). Net weight gain within each group was calculated.

2.4. Evaluation of sexual maturation

As a criterion for sexual maturation, the timing of preputial separation for male rats (beginning on PND 35) was assessed and each pup was weighed when these criteria were achieved.

2.5. Evaluation of fertility

At the end of the experiment, three animals from each treatment group were housed together with an untreated female rat, on two occasions. A vaginal smear from each paired female was collected on the four days following the pairing and the number of spermatozoa on it was counted. For the resulting litter, litter size, morbidity, and mortality were noted. Fertility of each male was calculated using the following formula:

Fertility (%) = {(number of pups in the male's litters)/(total number of pups) *100}

2.6. Animal euthanization

At PND80, male rats (n = 7 per group) were weighed, euthanized using an overdose of diethyl ether, trunk blood was collected and testicular and epididymis organs were sampled, weighed and processed for histological and sperm parameters.

2.7. Analysis of plasma biochemistry

Plasma samples, having been stored at −20 °C, were further analyzed for total cholesterol, triglycerides, HDL and LDL using AMP diagnostic kits (AMEDA labor diagnostic Gmbh, Austria) on a chemistry analyzer according to the manufacturer's instructions.

2.8. Sperm parameters

2.8.1. Sperm motility

To analyze sperm motility, the cauda epididymis was cut with a scissors and placed in 0.5 mL pre-warmed (at 37 °C) phosphate buffer saline (PH 7.3). A drop of nigrosin stain was added to it. 50 μL of this solution was put on a pre-warmed (at 37 °C) glass slide and inspected under a light microscope at 40 × magnification. At least 10 fields were observed, and 100 sperm per sample were analyzed for motility. Each sample was evaluated three times, after which the average sperm motility was used as the total sperm motility for a particular sample (Halvaei et al., 2012a).

2.8.2. Assessment of sperm viability

To assess sperm viability we used the eosin-nigrosin staining test. 25 μl of eosin-nigrosin dye was mixed with semen samples. 15 μl of this mixture was placed on a glass slide and dried at room temperature. Smears were examined under a light microscope (40 × magnification). Living spermatozoa remained unstained (white) whereas dead cells were stained red. The percentage of dead and alive spermatozoa was calculated by counting at least 100 sperm cells (Halvaei et al., 2012b).

2.8.3. Epididymal sperm count (ESC)

In order to determine the epididymal sperm count, the epididymis was cut into three small parts; caput, corpus, and cauda. The sections were minced in 1 ml physiological saline and the homogenate was incubated at 37 °C for 15 min. After incubation, 2–3 drops of nigrosine were added into the homogenate, of which ten μL were placed on a pre-warmed slide. A minimum of 10 fields were observed. Spermatozoa were counted in each of the three parts of the epididymis using a light microscope at 40 × magnification (Amin et al., 2012).

2.8.4. Daily sperm production (DSP)

Frozen testicular tissues were thawed at room temperature and the tunica albuginea was removed. The tissue was homogeneized in 5 mL NaCl (0.9%)/Triton X-100 (0.5%) solution using a rotor stator homogenizer (IKA-Werke, Staufen, Germany) for 30 s (Robb et al., 1978). The homogenate was diluted 5 times, transferred to a Neubauer chamber and 19th stage spermatids were counted under a light microscope at 40 × magnification. The average of at least three readings was taken as

the number of spermatids. To obtain the daily sperm production (DSP), the number of spermatids (an average of at least three readings of one sample) was divided by 6.3 (number of days the spermatids remain in seminiferous epithelium).

2.9. Histology

Testis and epididymis tissue samples were fixed in 10% formalin for 48 h. Dehydration was carried out at room temperature using ascending concentrations of alcohol, and shifting to xylene. After clearing, tissues were embedded in paraffin wax and five to seven μm thick sections were cut using a microtome (Thermo Fisher Scientific, UK). Sections were transferred to albumenized slides that were preheated to 37 °C. For complete stretching of tissues and removal of bubbles, slides were incubated overnight at 58 °C. Tissues were rehydrated in descending concentrations of alcohol, stained with hematoxylin-eosin stain and covered with a coverslip. The prepared slides were observed under a Leica microscope (New York, USA) equipped with a digital camera (Canon, Japan) at 40 × magnification. The percentage area covered by seminiferous tubules was calculated using ImageJ software as previously described by (Jensen, 2013).The area percentage was calculated by using the following formula

$$\% As = As \times 100/T$$

Where As is the area covered by seminiferous tubules, T is the total area of the field. The %age of the mean area was calculated between control and treated groups for comparison.In addition, the number of spermatogonia, spermatocytes and spermatids was counted from 50 seminiferous tubules per animal at 100 × magnification.

2.10. Hormonal analysis

Plasma levels of testosterone, LH, FSH, GH and cortisol were determined by Enzyme-linked immune Sorbent assay (ELISA) kits; for LH, FSH and testosterone (Reddot Biotech Inc. Canada), GH (Crystalchem, USA) and for cortisol (Calbiotech, Inc. USA) according to the instructions in the kit.

2.11. Statistical analysis

The results are stated as mean ± SEM. All statistical analysis was done by using 'lme4' (Bates et al., 2014) and 'easyanova' (Arnhold, 2013) packages for R statistical software version 3.2.5 (R Development Core Team, 2016). The effect of the treatment was analyzed using one-way ANOVAs ('ea1' function of 'easyanova') with each of the measured physiological/histological parameters as a response variable and furan concentration as the dependent categorical variable. Post-hoc differences were computed with the Tukey Honest Significant Differences (the R function 'TukeyHSD'). Plasma hormone concentrations of cortisol, plasma testosterone, LH, FSH and GH were correlated amongst each other using Pearson's correlations. We checked each model's residuals for normality using Shapiro-Wilk tests. The assumption of homogeneity of variances was assessed using Levene's test and additivity was verified using Tukey's test of additivity. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of furan on body weight gain, accessory organ weight and abdominal fat

All groups gained body weight from PND 1 to PND, but weight gain was significantly higher in animals treated with the highest dose of furan (20 mg kg^{-1} d^{-1}) in comparison with the control group (Table 1, P = 0.01). No significant difference was observed among other treated groups (Table 1). Weights of accessory reproductive organs (paired testis, paired epididymis, seminal vesicle, and prostate) were not significantly different among furan treatments (Table 1), although there was a tendency for increased weight in the high dose group. Weight of the abdominal fat pad was significantly higher in the high dose 20 mgkg^{-1}d^{-1}treated group (P = 0.02) and the same tendency (although not statistically significant) was observed in the 10 mg kg^{-1} d^{-1} group (P = 0.1,Table 1).

3.2. Effect of furan on plasma biochemical parameters, onset of puberty and fertility

Plasma concentrations of protein (P = 0.06), triglycerides (P = 0.001) and HDL (P = 0.001) were significantly reduced in the high dose group (20 mg kg^{-1} d^{-1}) compared to controls (Table 2). Plasma cholesterol and LDL levels showed significant (P = 0.06 and P = 0.001, resp.) increases in the 5, 10 and 20 mg kg^{-1} d^{-1} dose groups (Table 2). The day of preputial separation was not significantly different among furan treatment groups (P = 0.53; Table 2). We found a non-significant reduction in fertility in the high dose group (P = 0.07, Table 1), and there was also a non-significant dose-dependent reduction in fertility % with increasing furan exposure (P = 0.07, Table 2). No signs of morbidity and mortality were recorded in resultant pups.

3.3. Effect of furan on sperm parameters

A marked reduction in daily sperm production (DSP) was observed in response to furan treatment (Table 3). Compared to the control group, the 10 mg kg^{-1}d^{-1} and 20 mg kg^{-1} d^{-1} treatment groups showed a dose dependent decrease in DSP (P = 0.06 and P = 0.02 respectively, Table 3). Furan treatment was furthermore associated with a significant reduction in sperm viability (P = 0.003) and epididymal sperm count (P = 0.05; Fig. 1). A non-significant tendency for lower sperm motility was observed with increasing furan doses (P = 0.16). Epididymal sperm count (caput, corpus, cauda) was significantly lower among the groups treated with the highest doses (P = 0.09, P = 0.004 and P = 0.04 resp. in 5, 10, 20 mg kg^{-1} d^{-1} groups; Fig. 1).

3.4. Effect of furan after neonatal exposure on hormonal profile

Plasma testosterone concentration was reduced in the 10 (p = 0.06) and 20 mg kg^{-1} d^{-1} (p = 0.001) treated groups compared to the control group (Table 4). Similarly, significant reductions in plasma LH level (P = 0.01) and GH concentrations (P = 0.001) were observed in the groups receiving the two highest furan doses (Table 4). A non-significant reduction in FSH level was observed in the high dose group. Cortisol concentrations were significantly elevated in the two high dose groups (P = 0.001 and P = 0.01 in the 10 and 20 mg kg^{-1} d^{-1} groups; Table 4).

To investigate the relationship between plasma cortisol concentration and plasma testosterone, LH, FSH and GH concentrations, we correlated these hormone concentrations in a pairwise fashion (Table 5, Fig. 2). Results indicated that plasma cortisol concentration is negatively correlated with plasma testosterone (r = −0.385, P = 0.006) and GH (r = −0.5131, P < 0.01), while the relationship between cortisol and LH (r = −0.162, P = 0.261) and cortisol and FSH (r = −0.155, P = 0.281) was not significant. Moreover, testosterone and LH, as well as testosterone and GH correlated positively with each other (r = 0.379, P = 0.007 and r = 0.560, P < 0.001, resp.), while testosterone and FSH were not correlated (r = 0.119, P = 0.411). Finally, a positive correlation appeared between LH and FSH (r = 0.298; P = 0.03; Table 5 and Fig. 2).

3.5. Effect of furan on testicular histology

Analysis of testicular histology indicated a signficant reduction in

Table 1
Effects of neonatal subcutaneously exposure to furan (0, 1, 5, 10, 20 mgkg^{-1}d^{-1}) on body weight gain, accessory organ weight and abdominal fat pad weight in adult male rats.

Parameters	Control	Furan (mgkg^{-1}d^{-1})				Statistics
		1	5	10	20	
Body weight gain (g)	236.16 ± 6.01^{a}	237.1 ± 7.49^{ab}	240.7 ± 4.69^{ab}	238.1 ± 3.57^{b}	243.7 ± 8.87^{b}	P = 0.01 F = 3.83
Paired testis weight (g)	1.73 ± 0.05	1.70 ± 0.14	1.69 ± 0.09	1.71 ± 0.08	1.68 ± 0.06	P = 0.9 F = 0.04
Paired Epididymis weight (g)	0.94 ± 0.12	0.80 ± 0.09	0.76 ± 0.06	0.71 ± 0.10	0.65 ± 0.04	P = 0.60 F = 0.69
Prostate (g)	0.47 ± 0.06	0.46 ± 0.17	0.44 ± 0.19	0.47 ± 0.15	0.44 ± 0.08	P = 0.99 F = 0.01
Seminal Vesicle (g)	0.56 ± 0.06	0.55 ± 0.06	0.50 ± 0.19	0.48 ± 0.12	0.44 ± 0.14	P = 0.8 F = 0.27
Fat Pad (g)	1.24 ± 0.02^{b}	1.34 ± 0.03^{ab}	1.40 ± 0.09^{ab}	1.47 ± 0.11^{ab}	1.59 ± 0.09^{a}	P = 0.03 F = 2.96

Values are presented as Mean ± SE of mean (n = 7). P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P < 0.05) in the rows compared control with other treated groups.

Table 2
Effects of neonatal subcutaneously exposure to furan (0, 1, 5, 10, 20 mg kg^{-1} d^{-1}) on biochemical plasma parameters, puberty onset and fertility in adult male rats.

Parameters	Control	Furan (mg kg^{-1}d^{-1})				Statistics
		1	5	10	20	
Protein (mg 0.5g^{-1})	5.51 ± 0.20^{a}	5.46 ± 0.20^{a}	5.47 ± 0.06^{a}	5.16 ± 0.23^{ab}	4.88 ± 0.17^{b}	P = 0.03 F = 2.95
Cholesterol (mg dL^{-1})	40 ± 1.93	43.14 ± 2.66	42 ± 1.99	46.42 ± 1.88	46 ± 1.43	P = 0.06 F = 2.53
Triglyceride (mg dL^{-1}	53.28 ± 1.29^{a}	50.85 ± 1.80^{a}	44.57 ± 0.37^{b}	44.14 ± 1.72^{b}	44.42 ± 1.65^{b}	P = 0.001 F = 12.51
HDL (mg dl^{-1})	73 ± 1.16^{a}	72.14 ± 1.26^{a}	65.57 ± 1.50^{b}	65.71 ± 1.99^{b}	62.42 ± 2.25^{b}	P = 0.001 F = 12.47
LDL (mg dl^{-1})	29.54 ± 1.66^{b}	32.31 ± 1.11^{b}	39.54 ± 2.15^{ab}	34.94 ± 2.09^{b}	40.8 ± 2.33^{a}	P = 0.001 F = 7.75
Day of puberty onset	44.14 ± 0.51	44.42 ± 0.62	44 ± 0.54	43.57 ± 0.37	43.14 ± 0.75	P = 0.53 F = 0.79
No of pups born/female	8 ± 0.38	7.57 ± 0.37	7.85 ± 0.34	8 ± 0.31	6.71 ± 0.48	P = 0.07 F = 0.79
Fertility %	100 ± 4.80	98.24 ± 3.85	97.24 ± 4.69	94.64 ± 4.69	82.76 ± 5.72	P = 0.07 F = 0.79

Values are presented as Mean ± SE of mean (n = 7). P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P < 0.05) in the rows compared control with other treated groups.

the percentage area covered by seminiferous tubules in the two high treatment groups (Table 6; P = 0.01 and P = 0.002 for 10 and 20 mg kg^{-1} d^{-1}, resp.) while the area covered by interstitial spaces increased significant (P = 0.001) compared to the control group. The changes were in the same direction for the other variables measured (tubular and lumen diameter, and epithelial height) but were not statistically significant here (Fig. 3, Table 6).

3.6. Effect of furan on cells in seminiferous tubules

The number of spermatogonia present in the seminiferous tubules of the animals was significantly reduced in the groups exposed to the three

Table 3
Effects of neonatal subcutaneously exposure to furan (0, 1, 5, 10, 20 mg kg^{-1} d^{-1}) on sperm parameters in adult male rats.

	Control	Furan (mg kg^{-1}d^{-1})				Statistics
		1	5	10	20	
DSP (× 10^{6})	3.97 ± 0.23^{a}	3.75 ± 0.77^{a}	2.92 ± 0.311^{ab}	2.55 ± 0.22^{c}	2.37 ± 0.07^{c}	P = 0.01 F = 3.95
Sperm viability %	17.85 ± 1.70^{a}	14.28 ± 1.17^{ab}	13.14 ± 1.37^{ab}	12.71 ± 1.58^{ab}	9.28 ± 0.94^{b}	P = 0.003 F = 5.004
Sperm motility%	80.42 ± 1.92	79.14 ± 2.60	76.85 ± 1.14	74.57 ± 1.48	76.28 ± 1.30	P = 0.16 F = 1.73

Values are presented as Mean ± SE of mean (n = 7). P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P < 0.05) in the rows compared control with other treated groups.

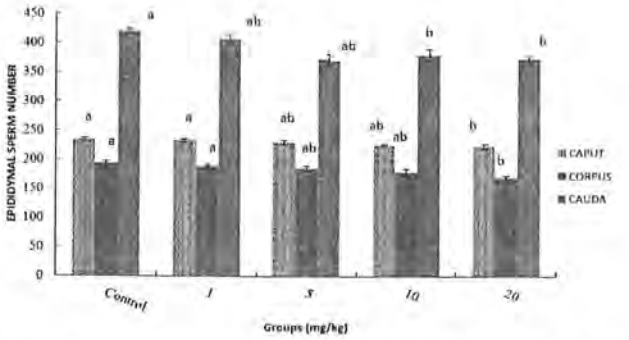

Fig. 1. Effects of neonatal exposure to furan (0, 1, 5, 10, 20 mgkg^{-1}d^{-1}) on epididymal sperm counts (in caput, corpus and cauda parts) in adult male rats. Values represent mean ± SE of mean (n = 7). Letters above bars depict values that are significantly different (P < 0.05) between different doses within the same epididymal part.

highest doses of furan (Table 7; P = 0.003, P = 0.001 & P = 0.001 in 5, 10, 20 mg kg^{-1} d^{-1} doses, resp.). Similarly, a significant (p = 0.001) reduction in a number of spermatocytes was also observed in the two high dose groups (10 mgkg^{-1} and 20 mgkg^{-1}d^{-1}) as compared to control. In addition, the number of spermatids was significantly reduced in the 20 mgkg^{-1}d^{-1} dose group (P = 0.04; Table 7).

3.7. Effect of furan on epididymis histology

No significant morphometric alterations were detected in the histology of epididymis caput tissue of adult rats. There was however a trend for a reduction in tubular and lumen diameters, and increase in epithelial height in the high dose group (Table 8, Fig. 4).

A similar pattern was observed in the cauda epididymis (Table 9, Fig. 5) although here, epithelial height was significantly increased (P = 0.05). No significant difference was recorded in other parameters of epididymis in treated groups when compared to control. No differences between treatment groups were observed for epididymis corpus histology.

4. Discussion

Few animal studies have investigated the possible endocrine disrupting effects of furan. To our knowledge, the current study is the first study that exposed rats to furan at the neonatal stage of life, and estimated the effects on male reproductive system in adulthood. Neonatal male rats were subcutaneously injected with four different furan doses (1, 5, 10 and 20 mgkg^{-1}d^{-1}in 50 μL corn oil), in order to investigate the effects in adult male rats (PND80). We find clear evidence that exposure to the two highest doses of furan (10 and 20 mg kg^{-1} d^{-1}) broadly and consistently affects the development of the male reproductive system, ultimately compromising male fertility in adulthood. The most prominent effects we observed were dose dependent increases in body weight, decreased plasma testosterone, LH and GH, increased cortisol levels, and decreased sperm counts associated with altered testicular and epididymal histology.

Elevated weight gain has been observed in an earlier study where rats were exposed to furan from the weaning period to post-puberty (Karacaoğlu and Selmanoğlu, 2010) and in suckling mice after furan treatment (Blagburn et al., 1998). The mechanism of how furan affects body weight gain is unknown, but one can speculate that it could be the result of a direct effect on nutrition uptake, or an indirect effect via the observed changes in plasma hormones (Mårin et al., 1992). has suggested that cortisol can cause obesity, an idea that has been further developed by later studies (Abraham et al., 2013; FTC, 2004). Also low testosterone levels are associated with adiposity resulting from to metabolic impairments (Fui et al., 2014), while testosterone replacement therapy instigated a reduction in fat mass and prominent increase in fat-free mass in both hypogonadal and normal men (Cox et al., 1999; Evans, 2000; Gulve and Spina, 1995). Rasmussen (2010) reported that GH deficiency is related to increased fat mass and abdominal fat mass accumulation (Rasmussen, 2010). Hence, the increase in weight gain and abdominal fat mass we found in the present study in the group receiving the highest dose (20 mg kg^{-1} d^{-1}) may well have been caused by the elevation of cortisol levels and the decreased level of testosterone, LH and GH.

The relationships we observed between plasma hormone levels furthermore fit well in with earlier studies reporting negative relationships between plasma cortisol vs. testosterone or GH (Wemink et al., 1990). Indeed, many components of the gonadal axis are downregulated by plasma glucocorticoids, either by affecting hypothalamus and pituitary functions, or by affecting the responsiveness of target tissues to gonadal hormones (Borges et al., 1997; Thakore and Dinan, 1994). Increased cortisol secretion has been associated with a decreased production of sex steroids and GH in a multitude of studies (Björntorp, 1995; Burguera et al., 1990; Chen et al., 1997; Liening and Josephs, 2010; Tsigos and Chrousos, 2002; Viau, 2002). High doses of glucocorticoids inhibited testicular Leydig cell function in rats (Bambino and Hsueh, 1981). In humans, exposure to cortisol caused a marked decrease in testosterone production (Cumming et al., 1983). A decrease in the testosterone production in adulthood is a recurrent finding in studies exposing rats to heat-induced food toxicants (El-Akabawy and El-Sherif, 2016; Karacaoğlu and Selmanoğlu, 2010; Salian et al., 2009). Also endocrine disruptors such as PAHs and PCBs, as well as lead and cadmium cause elevated cortisol levels and decreased GH

Table 4
Effects of neonatal subcutaneously exposure to furan (0, 1, 5, 10, 20 mgkg^{-1}d^{-1}) on hormonal profile in adult male rats.

	Control	Furan (mg kg^{-1}d^{-1})				Statistics
		1	5	10	20	
Testosterone (ng ml^{-1})	4.45 ± 0.20^{a}	4.28 ± 0.025^{a}	4.13 ± 0.17^{a}	3.83 ± 0.16^{ab}	3.24 ± 0.15^{b}	P = 0.001 F = 8.95
LH (ng ml^{-1})	1.79 ± 0.04^{a}	1.74 ± 0.04^{ab}	1.69 ± 0.03^{ac}	1.63 ± 0.03^{bc}	1.59 ± 0.04^{c}	P = 0.01 F = 3.41
FSH (miUml^{-1})	1.03 ± 0.09	1.02 ± 0.005	0.96 ± 0.10	0.89 ± 0.08	0.80 ± 0.02	P = 0.1 F = 1.57
GH (ng ml^{-1})	210.7 ± 1.75^{a}	203.6 ± 2.13^{ab}	197.8 ± 2.28^{bc}	190 ± 3.62^{cd}	181.6 ± 3.90^{d}	P = 0.001 F = 15.45
Cortisol (ng ml^{-1})	39.8 ± 0.89^{c}	41.2 ± 0.94^{bc}	44.1 ± 0.88^{ac}	44.4 ± 1.56^{ab}	46.5 ± 1.05^{a}	P = 0.001 F = 5.94

Values are presented as Mean ± SE of mean (n = 7). P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P < 0.05) in the rows compared to control with other treated groups.

Table 5
Summary of the Pearson's correlations between plasma cortisol, testosterone, LH, FSH and GH in adult male rats neonatally exposed to furan (0, 1, 5, 10, 20 mg kg^{-1} d^{-1}).

Parameters	Correlations				
	Cortisol (ng ml^{-1})	Testosterone (ng ml^{-1})	LH (ng ml^{-1})	FSH (mlUml^{-1})	GH (ng ml^{-1})
Cortisol (ng ml^{-1})	r = 1				
Testosterone (ng ml^{-1})	r = −0.385**	r = 1			
	P = 0.006				
LH (ng ml^{-1})	r = - 0.162	r = 0.379**	r = 1		
	P = 0.261	P = 0.007			
FSH (mlUml^{-1})	r = − 0.155	r = 0.119	r = 0.298*	r = 1	
	P = 0.281	P = 0.411	P = 0.036		
GH (ng ml^{-1})	r = −0.513**	r = 0.560**	r = 0.261	r = 0.102	r = 1
	P = 0.000	P = 0.00	P = 0.250	P = 0.482	

Pearson's correlation and sets significant relation; Pearson's correlation is shown with r whenever significant by P -value.

levels in fish and mammals (Sajjad et al., 2018; Tan et al., 2007; Tort et al., 1996; Zimmer et al., 2009). This suggests that the growth of the reproductive organs can be disturbed by reduced testosterone, which in turn could be driven by increased cortisol levels.

We found a consistent decrease in both the quality and number of sperm from males neonatally exposed to the two highest doses of furan. A significant reduction in sperm viability and motility was observed in highest dose treated group, and daily sperm production and epididymis (caput, corpus and cauda) sperm counts were reduced in a dose-dependent manner. These findings may be explained by the lower testosterone and LH concentrations. The elevated cortisol levels may have contributed to the inhibition of spermatogenesis, disturbance of spermiation and impairment of sperm quality (Castranova et al., 2005; Pressman et al., 2018). Also furan exposure at the weaning stage of life resulted in spermatogenic impairment and apoptosis in germ cells and Leydig cells which was associated with lower LH and testosterone levels (Karacaoğlu and Selmanoğlu, 2010).

Histological analysis of the testis showed a significant decline in the seminiferous tubules' diameter, epithelial height and area covered by the seminiferous tubule, associated with exposure to furan, and wider lumen and larger spaces in the interstitium. In the groups receiving the highest doses, prominent multilayered spermatogonia were recorded, while seminiferous tubules lumen was not heavily packed with elongated spermatozoa. These findings are in accordance with earlier reports of apoptosis in germ cells and Leydig cells after furan exposure (Karacaoğlu and Selmanoğlu, 2010). In contrast to the testes, morphometric findings of the epididymis showed few significant alterations. The only change observed was in the caput epididymal diameter,

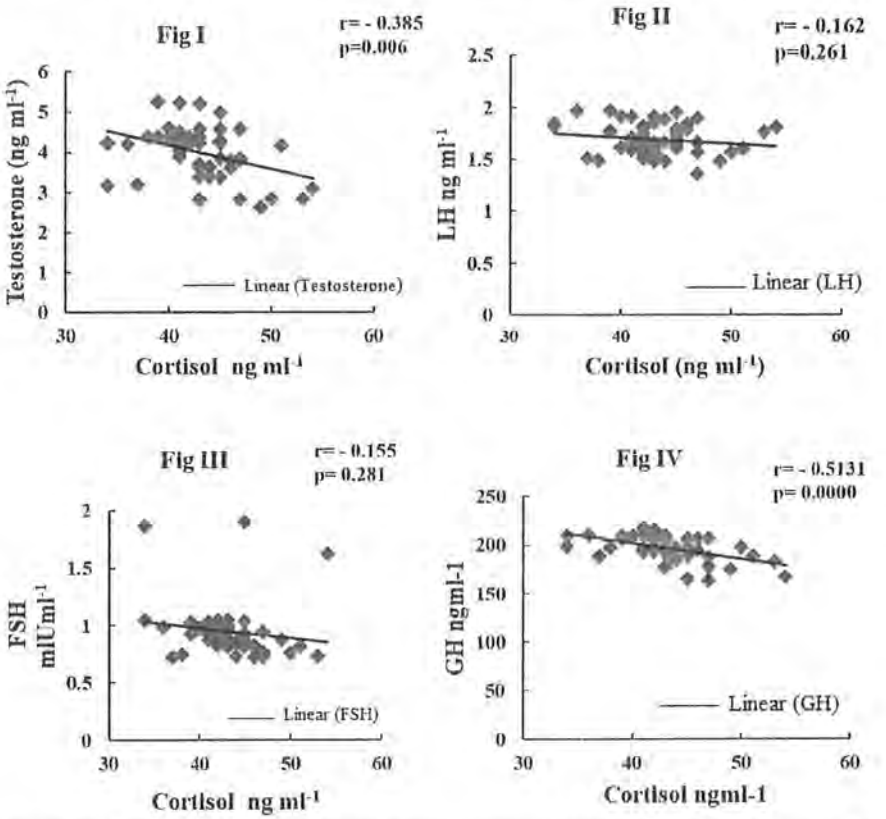

Fig. 2. Plasma testosterone (I), LH (II), FSH(II) and GH (IV) as a function of plasma cortisol concentration in adult male rats neonatally to furan (0, 1, 5, 10, 20 mgkg^{-1}d^{-1}). Pearson's correlation coefficient (r) and P values are provided in the top right of each graph.

Table 6
Effects of neonatal subcutaneously exposure to furan (0, 1, 5, 10, 20 mgkg^{-1}d^{-1}) on testicular tissue histopathology in adult male rats.

Parameters	Control	Furan (mg kg^{-1}d^{-1})				Statistics
		1	5	10	20	
Area of seminiferous tubule %	89.18 ± 0.63^{a}	87.05 ± 0.78^{ab}	86.11 ± 0.90^{ab}	84.97 ± 1.26^{b}	84.35 ± 0.94^{b}	P = 0.002 F = 4.25
Area of interstitium %	12.66 ± 0.58^{a}	13.89 ± 0.52^{a}	14.56 ± 0.49^{b}	17.20 ± 0.59^{bc}	17.84 ± 0.56^{c}	P = 0.001 F = 16.18
Seminiferous tubule diameter (μm)	234.26 ± 6.82	227.43 ± 6.73	223 ± 10.49	223.42 ± 7.38	218.88 ± 9.65	P = 0.7 F = 0.49
Epithelial height (μm)	63.23 ± 2.51	60.04 ± 2.87	61.84 ± 2.88	58.11 ± 2.42	58.51 ± 2.56	P = 0.60 F = 0.68

Values are presented as Mean ± SE of mean (n = 7). P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different ($P < 0.05$) in the rows compared to control with other treated groups.

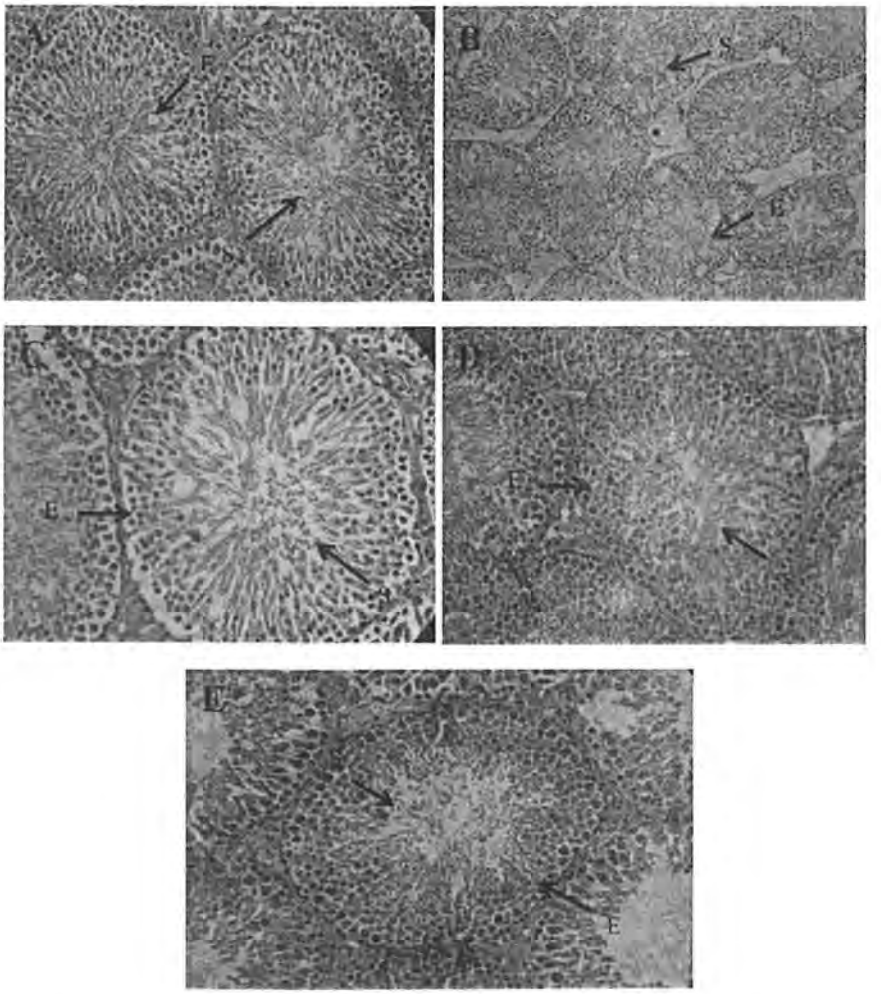

Fig. 3. Photomicrograph (40 × magnification) of seminiferous tubules (hematoxylin-eosin stain) of adult male rats exposed to different doses of furan at neonatal stage. (A) control showing regularly arranged tubules, lumen filled with spermatids with normal germ cells; (B) 1 mg kg^{-1} d^{-1} showing normal spermatogenesis with lumen filled with mature spermatozoa; (C) 5 mg kg^{-1} d^{-1} displays normal morphology with a slightly widened lumen; (D) 10 mg kg^{-1} d^{-1} group showing increased lumen diameter and normal epithelial height; (E) 20 mg kg^{-1} d^{-1} showing a thin degenerated epithelium with empty lumen. Letters indicate spermatozoa (S), and epithelium (E).

which showed a dose-dependent reduction.

We observed increases in plasma cholesterol and LDL while triglyceride (TG) and HDL were decreased in the groups receiving the two highest doses of furan, findings that are in line with the observed increases in body mass and abdominal fat. The increased production of fatty acids leads to a reduction in plasma HDL and elevation in plasma cholesterol concentrations, which can ultimately result in liver dysfunction. Lipoproteins (HDL and LDL) are therefore considered sensitive biomarkers of liver function (Rawi et al., 2012). TG/HDL ratio is clinically used for the detection of apparently healthy individuals with cardiovascular or metabolic impairments (Murguía-Romero et al., 2013). The lowest levels of HDL are detected in patients with fasting chylomicronæmia and in hypertriglyceridemic subjects, suggesting an inverse relationship between the metabolism of triglyceride and HDL (Schaefer et al., 1978). However, the present study detected a decrease in both tryglyceride and HDL associated with furan treatment, which could be explained by the observation that plasma lipoprotein levels often remain low during the early stages of development (Robinson and

Table 7
Effects of neonatal subcutaneously exposure to furan (0, 1, 5, 10, 20 mgkg^{-1}d^{-1}) on different types of cells in seminiferous tubules of testes in adult male rats.

Cells number	Control	Furan (mg kg^{-1}d^{-1})				Statistics
		1	5	10	20	
Spermatogonia (n)	61.4 ± 1.023^{a}	58.6 ± 0.75^{ab}	56.6 ± 0.80b^{c}	55.64 ± 0.98^{bc}	53.08 ± 1.06^{c}	P = 0.001 F = 11.21
Spermatocytes (n)	81.08 ± 1.165^{a}	78.2 ± 1.50^{ab}	77.48 ± 1.137^{ab}	77.12 ± 0.83^{ab}	75.58 ± 1.06^{b}	P = 0.01 F = 3.03
Spermatids (n)	257.52 ± 2.14	255.84 ± 2.24	253.56 ± 2.34	253.32 ± 1.92	248.8 ± 2.39	P = 0.06 F = 2.20

Values are presented as Mean ± SE of mean (n = 7). P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P < 0.05) in the rows compared control with other treated groups.

Seakins, 1963). An alterative explanation for the observed changes in plasma lipoproteins stems from the observation that a number of liver toxins have been reported to inhibit the release of hepatic triglyceride into the bloodstream. Acrylamide, for example, also causes changes in lipoproteins in the same direction (Rawi et al., 2012).This would explain the observed low plasma triglyceride concentration and could also have led to a the buildup of triglyceride in the liver (Robinson, 1973), which has been associated with liver damage and fibrosis in obese mice (Yamaguchi et al., 2007).A third possible explanation for the observed elevated plasma LDL levels could be the overactivation of LDL receptors, as first proposed by Selmanoğlu et al. (2012). In the latter study male rats were exposed to furan, which also resulted in elevated plasma LDL levels. LDL receptors enable cholesterol to enter normal body cells. Once attached to LDL receptors on the hepatocytes, LDLs release their cholesterol and triglycerides. Elevated cholesterol in the cells suppresses the formation of new LDL receptors, resulting in decreased transport of LDL cholesterol into the cells (Elaine, 2009). Excess levels of free cholesterol inhibit cholesterol and LDL receptor synthesis, thus reducing LDL uptake which promotes cholesterol storage. Restricting the LDL uptake and non-functioning of its receptors enhance the serum cholesterol levels (Linda, 2012).

Also the reduction in plasma total protein levels can be regarded as evidence for a reduction in liver function, as previously reported by (Alam et al., 2017; Karakilcik et al., 2004).About 6%–7% of the blood plasma proteins, including albumin, fibrinogen and prothrombin are synthesized in the liver (Osserman and Takatsuki, 1963). According to previous studies, hypoalbuminemia is associated with advanced chronic liver diseases (Koneri et al., 2008). Liver damage could occur via peroxidation of polyunsaturated fatty acids which are detrimental to cellular homeostasis, through the formation of aldehydes. Aldehydes impair nucleotide and protein synthesis, increase production of the pro-inflammatory cytokines TNF-α, and promote an influx of inflammatory cells into the liver, leading to collagen deposition, hepatic injury and inflammatory response (Esterbauer et al., 1991; Yamauchi et al., 2003).

Importantly, we observed a dose-dependent decline in fertility (number of pups conceived) in male rats neonatally exposed to furan. This finding can be adequately explained by the lower testosterone levels that arguably resulted in lower sperm numbers and quality, which were produced by testes showing clear histological abnormalities. We find no evidence that furan exposure caused a delay in the onset of puberty (as determined from preputial separation), which has been observed for ethynylestradiol, among other EDCs (Yoshimura et al., 2005).These results are in accordance with previous studies in which genistein exposure showed no effects on the onset of puberty, but decline in the number of pups produced (Nagao et al., 2001).

5. Conclusion

We find clear evidence that exposure to the two highest doses of furan (10 and 20 mg kg^{-1} d^{-1}) broadly and consistently affects the development of the male reproductive system, which ultimately lead to a reduction in fertility. Our results are in line with previous studies on furan exposure at the weaning stage, and highlight that developmental exposure to furan can cause organizational effects on the development of male reproductive systems. Considering that furan is detected in infant food and baby milk formulas at concentrations in the ng L^{-1} range, this study provides important preclinical data on the minimal furan dose at which endocrine disruption can occur. Our results also warrant investigation of the reproductive system of female rats, as well as a screening for other endocrine effects unrelated to reproductive systems.

Table 8
Effect of neonatal subcutaneous exposure to furan (0, 1, 5, 10, 20 mgkg^{-1}d^{-1}) on caput epididymis tissue histopathology in adult male rats.

Parameters	Control	Furan (mg kg^{-1}d^{-1})				Statistics
		1	5	10	20	
Tubule diameter (μm)	354.4 ± 8.33	350.08 ± 7.26	347.28 ± 8.12	339.76 ± 9.83	343.9 ± 10.04	P = 0.80 F = 0.40
Lumen diameter (μm)	255.28 ± 7.83	252.14 ± 7.27	253.36 ± 8.05	246.6 ± 9.31	237.84 ± 6.32	P = 0.51 F = 0.81
Epithelial heights (μm)	31.40 ± 0.96	29.00 ± 0.90	29.47 ± 1.02	28.20 ± 0.76	29.16 ± 0.82	P = 0.14 F = 1.73
Epithelium % age	39.25 ± 1.20	36.25 ± 1.13	36.84 ± 1.28	35.25 ± 0.95	36.46 ± 1.03	P = 0.143 F = 1.73
Lumen% age	60.74 ± 1.20^{b}	63.74 ± 1.13^{ab}	63.15 ± 1.28^{a}	64.74 ± 0.95^{ab}	63.53 ± 1.03^{ab}	P = 0.03 F = 2.62

Values are presented as Mean ± SE of mean (n = 7).P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different (P < 0.05) in the rows compared to control with other treated groups.

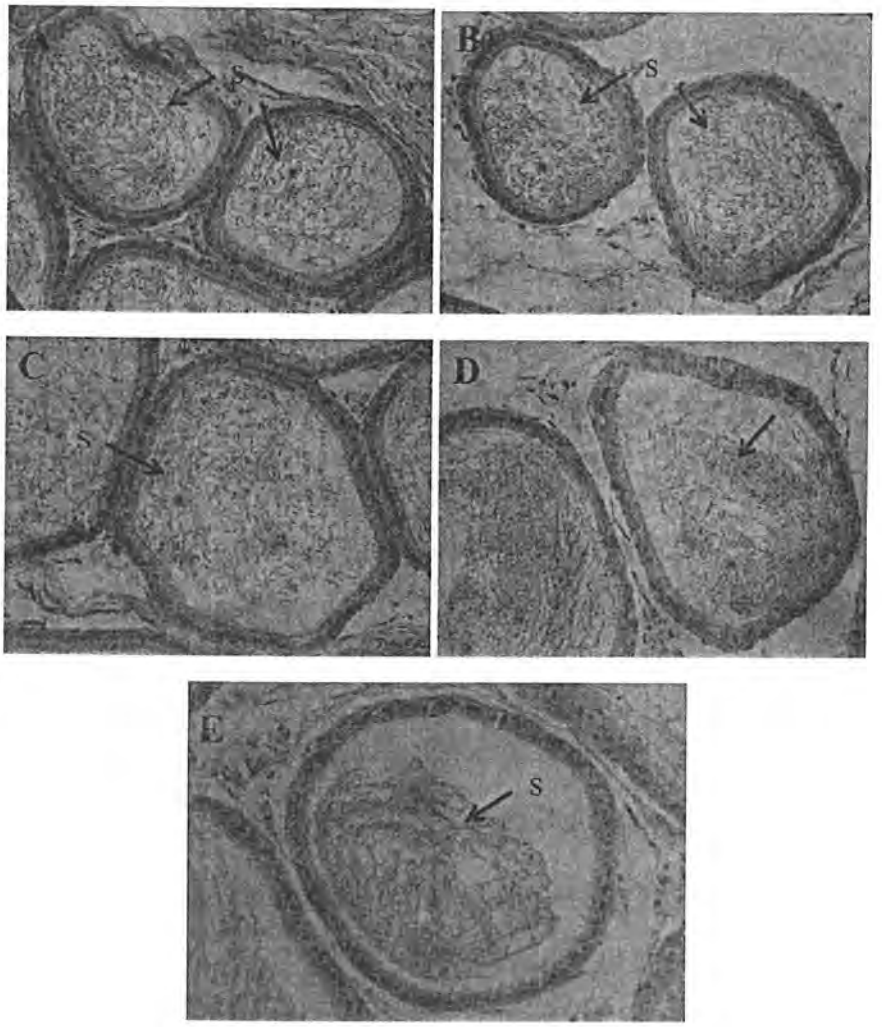

Fig. 4. Photomicrograph (40X magnification) of caput epididymis region (hematoxylin-eosin stain) of adult male rats exposed to different doses of furan at neonatal stage. (H&E, 40X) from: (A) Control group; with normal morphology of caput epididymal cells having thin pseudostratified epithelium lumen heavily filled with spermatozoa, (B) 1 $mgkg^{-1}d^{-1}$group; with thin epithelium-lined with stereocilia, (C) 5 $mgkg^{-1}d^{-1}$group; showing normal spermatozoa in lumen (D) 10 $mgkg^{-1}d^{-1}$group; showing increase in pseudostratified epithelium and decrease in concentration of spermatozoa, (E) 20 $mgkg^{-1}d^{-1}$group; Slight reduction in spermatozoa. Letters indicate spermatozoa (S), and epithelium (E).

Declaration of interest

All the authors who have a major contribution in this research articles have no declarations of interest.

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Table 9
Effects of neonatal subcutaneously exposure to furan (0, 1, 5, 10, 20 $mgkg^{-1}d^{-1}$) on caudal epididymis tissue histopathology in adult male rats.

Parameters	Control	Furan (mg $kg^{-1}d^{-1}$)				Statistics
		1	5	10	20	
Tubule diameter (μm)	454.48 ± 7.13	453.22 ± 8.91	449.24 ± 9.30	449.42 ± 10.09	445.7 ± 10.54	P = 0.9 F = 0.14
Lumen diameter (μm)	415.2 ± 10.99	417.7 ± 8.24	413.3 ± 7.83	409.14 ± 9.57	404.98 ± 11.96	P = 0.90 F = 0.26
Epithelial heights (μm)	27 ± 1.29^{a}	25.03 ± 1.27^{ab}	25.96 ± 1.40^{ab}	23.14 ± 1.69^{ab}	22.03 ± 1.29^{b}	P = 0.05 F = 2.31
Epithelium % age	34.14 ± 1.61^{a}	31.29 ± 1.58^{ab}	32.45 ± 1.75^{ab}	28.93 ± 2.12^{ab}	27.54 ± 1.61^{b}	P = 0.05 F = 2.35
Lumen% age	65.85 ± 1.61^{b}	68.70 ± 1.58^{ab}	67.54 ± 1.75^{ab}	71.06 ± 2.12^{ab}	72.45 ± 1.61^{a}	P = 0.05 F = 2.30

Values are presented as Mean ± SE of mean (n = 7).P and F value in the rows from ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with furan treated groups. Mean with different superscript are significantly different ($P < 0.05$) in the rows compared to control with other treated groups.

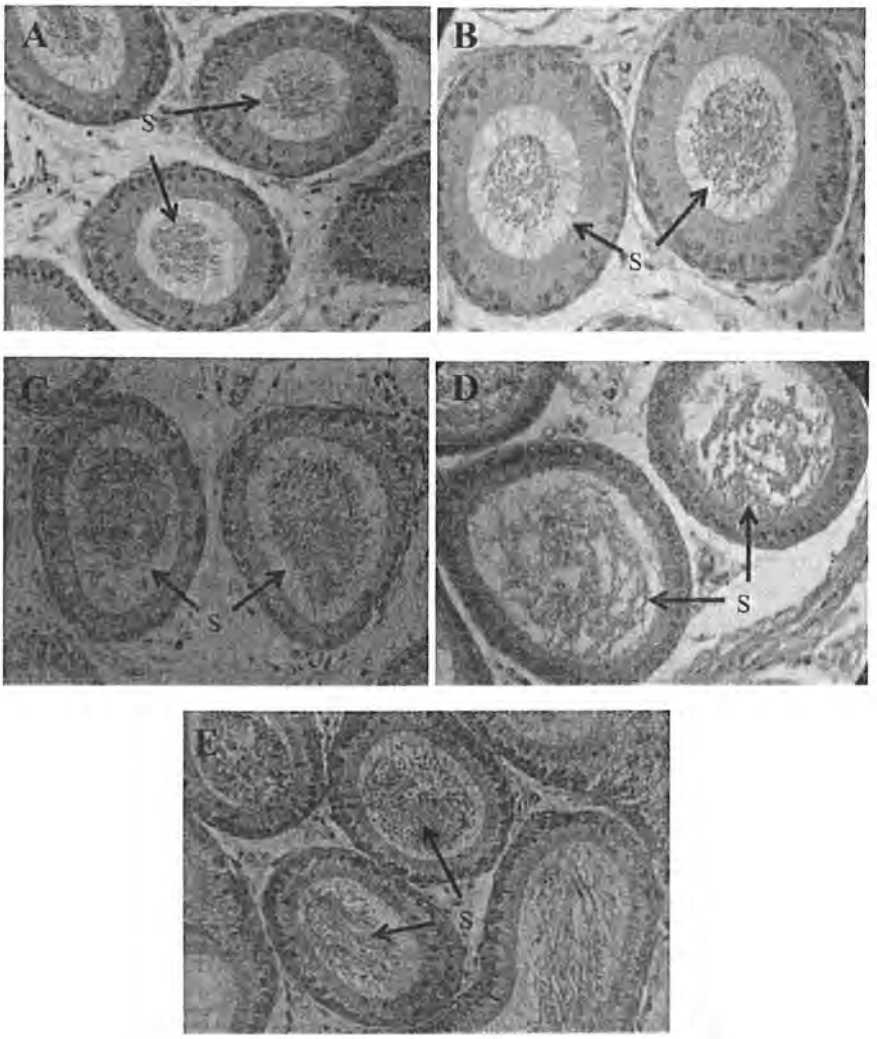

Fig. 5. Photomicrograph (40 × magnification) of cauda epididymis region (hematoxylin-eosin stain) of adult male rats exposed to different doses of furan at neonatal stage. (A) Control; exhibiting normal morphology of cauda epididymis; lumen filled with mature sperms, (B) 1 $mgkg^{-1}d^{-1}$ group; showing normal morphology like control. (C) 5 $mgkg^{-1}d^{-1}$group; showing slight changes in morphology of tubules, surrounded by stroma. (D) 10 $mgkg^{-1}d^{-1}$group; lumen has little number of spermatozoa. (E) 20 $mgkg^{-1}d^{-1}$group; showing a further increase in epithelial height and little lumen sperm concentration.Letters indicate spermatozoa (S), and epithelium (E).

Sweden for language and grammar check of the research article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.05.020.

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Effects of endocrine disruptor furan on reproductive physiology of Sprague Dawley rats: An F1 Extended One-Generation Reproductive Toxicity Study (EOGRTS)

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Abstract

The present study investigated the reproductive toxicity of furan in an Extended One-Generation Reproductive Toxicity Study in rats. Sprague Dawley F0 weaning rats (30 per sex per group) were exposed to furan orally at 0, 1, 2.5, 5, and 10 mg kg^{-1} for 10 weeks (males) and 2 weeks (females) and then mated. Results of F0 indicated that in the furan-treated groups (5 mg kg^{-1} and 10 mg kg^{-1}), body weight (bw) gain decreased during prebreed and gestational period while increased during lactation periods. F0 animals prebreeding exposure resulted in head tilt and foot splay at 10 mg kg^{-1}. Number of live pups at birth were decreased ($p < 0.001$) at 10 mg kg^{-1}. At postnatal day (PND) 70, a significant ($p = 0.03$) decrease in testosterone levels of male rats and estrogen levels of female rats ($p = 0.05$) was observed in 10 mg kg^{-1} furan-treated group in F1 generation. Luteinizing hormone, follicle-stimulating hormone, and progesterone levels were also reduced, but their reduction was not statistically significant in all groups. In higher dose furan group (10 mg kg^{-1}), testicular and ovarian weights were reduced in F1 generation at PND 70, with decreased daily sperm production ($p = 0.01$) and disturbed estrous cyclicity ($p < 0.01$). Some histopathological changes were also observed in testis and ovaries in groups whose parents were previously exposed to 10 mg kg^{-1} bw of furan group. Based on the above results, it is suggested that exposure to food-based contaminant furan induced remarkable changes in the F0 (parental stage) and F1 (offspring, pubertal, and adult stage) generations of Sprague Dawley rats.

Keywords

Furan, puberty, Extended One-Generation Reproductive Toxicity Study (EOGRTS), histopathology

Introduction

Furan (C_4H_4O) is a colorless volatile organic compound that is used in several industries and is also found in various common food products that undergo excessive heat treatment, such as canned and jarred foods, coffee, sauces, soups, baby foods, infant formula, and beverages.[1–5] Furan is also present in the environment, as it is the main constituent of cigarette smoke, wood smoke, and exhaust gases from engines.[6] Because of the toxic nature of furan and its excessive daily intake in humans through diet and air, it has become a matter of concern. The US Food and Drug Administration has reported the presence of

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furan in food items in various surveys.[1,7] The European Food Safety Authority also reported that among adults, prepared coffee is the main source of furan.[8] The International Agency for Research on Cancer has classified furan as a possible human carcinogen (group 2B).[9] Previous observations reported that furan caused various types of cancer and has an adverse effect on the biological system of humans and animals.[9–12] Furan has also been known as hepatoxic and carcinogenic in the rats and mice approved by National Toxicology Program.[13]

Furan needs to be removed from consumer products because of its negative effects on human health.[6] Various toxicants are dangerous to cell membranes since they cause increase in lipid peroxidation, production of reactive oxygen species (ROS), and DNA damage in the cells.[5,11,14] Furan can easily pass through biological membranes due to its low polarity and acts as an endocrine disruptor as it has been reported previously that exposure to different concentrations of furan resulted in the disturbance of the reproductive neuroendocrine system in rats.[5] It has been previously reported that furan is cytotoxic, genotoxic, and an apoptotic inducer.[15,16] Previous literature on furan reported that its exposure causes reproductive impairment by disturbing reproductive organ growth; spermatogenesis; testosterone (T), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) secretion; and reproductive performances.[5,6,16] It has been previously reported that hazardous environmental toxicants can bring variations in the signaling pathways of cells and defensive and protective systems of the antioxidant levels of the cells by affecting physiological conditions.[12,17] Furan caused significant changes in histological structure of cells, malondialdehyde levels, antioxidant enzyme activities, and caused DNA damage in lymphocytes and sperm cells by producing ROS.[6,11,12] Adverse effect of furan has been tested for a variety of reproductive parameters, and prominent effects were reported in the reproductive systems of males and females.[11,18–20] However, a study was conducted on Taiwanese boys whose mothers used contaminated (with polychlorinated biphenyls (PCBs) and furan) rice oil that resulted in smaller size of penis in comparison with age-matched control boys.[21] Similarly, Guo et al. reported that sperms of prenatally exposed young men with PCBs and furan have abnormal sperm morphology, reduced sperm motility, and also reduced ability of sperm to enter hamster oocytes.[22] Tyla et al. observed the reproductive toxicity of food toxicant acrylamide in the next two generation of Fisher (F344) rats.[23] Furan was also reported to occur in the morning urine samples of humans in different concentrations.[24]

Previously, many studies revealed the reproductive toxicity of furan in adult rats. Thus far, no information is available in the literature about the toxic effects of furan in the next generation. There is a considerable debate whether furan is a toxicant in the next generation. The current study was designed with the aim to study the Extended One-Generation Reproductive Toxicity Study (EOGRTS) across different life stages (parental, offspring, and adults). The main reason for this EOGRTS was to assess the possible effects of furan on F0 (parental) reproduction and F1 (first generation) offspring growth and development with calculations of endocrine function following exposure during early period of development. This EOGRTS design was performed in compliance with Environmental Protection Agency (EPA; Organisation for Economic Co-operation and Development (OECD) #443) testing guidelines and under EPA Good Laboratory Practice regulations.[25–29] In view of these concerns, we performed a frequent oral dose-based EOGRTS in Sprague Dawley rats to find out the potential toxic effects of furan on reproductive performance of rats in F0 (parental) and F1 (first generation).

Materials and methods

Animals

Chemicals and animals. Furan (Catalog no.185922, 99% purity) was purchased from Sigma–Aldrich, St Louis, Missouri, USA. Sprague Dawley rats (postnatal day (PND) 35) were collected from the Animal House Facility of Department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan. The rats were housed in steel cages. Prior to the start of the experiment, standard colony conditions were maintained accordingly.[6,20,30]

Ethical considerations

Animal handling and subsequent euthanization were done according to the guidelines provided by the "Ethics Committee" of the Department of Animal Sciences, Faculty of Biological Sciences, on care and use of animals for scientific research (Ref No.As-2018/431). Animal handling and euthanization protocols used currently were according to the European

Union guidelines for human use of animals for experimental purposes.

Study design

By following OECD 443, an EOGRTS was performed and additional analyses at the age of puberty was also performed. The animals were scattered among five dose groups for better assessment of dose–response relations. Male rats were exposed to furan 70 days prior to mating and female rats were exposed 14 days prior to mating to cover at least one full spermatogenic cycle or two estrogenic cycles. The first control group received a daily oral dose of corn oil (50 µL), while second, third, fourth, and fifth group received furan by gavage administration (1, 2.5, 5, and 10 mg kg^{-1} in 50 µL corn oil, respectively) for 70 days (male rats) and 14 days (female rats). Dose time was chosen according to the standard protocol 443 of the OECD (2011). Different doses of furan were selected to assess the dose-dependent EOGRTS exposure response. Furan doses used in this experiment were selected on the basis of doses used in previous studies on furan and acrylamide with some slight modifications in concentration[6,16,20,31,32] and OECD guideline #408 (doses can be used according to the relevant substance if doses have not been previously reported). In male rats, dose exposure continued during mating, and in female dams, dose exposure continued during pregnancy and lactation. Animals were housed in couples for mating, avoiding sibling mating. Pregnant females were single housed. Presence of sperm in vaginal smear or in the dams' vaginal plug was considered as gestational day (GD) 0 or day 0 of pregnancy. On GD 0, 6, 13, and 20, mated females were weighed. Each female was shifted to a shoe-box cage with bedding on GD 18. On the beginning of GD 20, females were monitored twice daily, for verification of littering. Male rats were monitored during prebreed and mating period and female rats during prebreed, gestation, and lactation period with a complete physical examination. Also, the number of living and dead pups, their weights, sex ratio, anogenital distance (AGD), nipple retention (NR), and survival rate were recorded. Litter size was not uniform. During the lactation period, the mortality, the time to vaginal opening, and preputial separation in pups were recorded. F1 females were housed individually, while F1 males had to be housed in small groups of five to six animals due to space limits. All the reproductive parameters of F1 males and females were documented. At around 10 weeks of age, F1 animals were marked for reproductive tests (hormonal profile, body weight (bw), and histopathology).

Formulas for indices:

$$\text{Mating index (female)}(\%) = \frac{\text{No. of plug/sperm positive}}{\text{No. of paired}} \times 100,$$

$$\text{Mating index (male)}(\%) = \frac{\text{No. of males impregnating}}{\text{No. of males paired}} \times 100,$$

$$\text{Fertility index}(\%) = \frac{\text{No. of pregnant}}{\text{No. of paired}} \times 100,$$

$$\text{Fecundity index}(\%) = \frac{\text{No. of pregnant}}{\text{No. of plug/sperm positive}} \times 100,$$

$$\text{Gestational index}(\%) = \frac{\text{No. of live litters, PND 0}}{\text{No. of total litters, PND 0}}.$$

Blood and tissue collection. At PND 70, male rats (n = 10 per group) and female rats (n = 10 per group) were weighed and blood samples were collected using cardiac puncture for hormonal analysis, while testicular, epididymis, prostrate, seminal vesicles, uterus, and ovarian organs were sampled, weighed, and processed for histological and sperm parameter.

Body weight gain. On PND 70, animals were weighed and mean bw gain was attained.

Determination of AGD, NR, and puberty onset. On PND 1, number of male and female pups were totaled, and the weight of each pup was recorded. The pups were examined for deformities, and in all pups, the AGD was examined in an ocular stereomicroscope. All the pups were weighed continuously on PND 0, PND 4, PND 7, and PND 14. On PND 14, male pups were inspected for number of NRs. Puberty in male and female pups was examined through the appearance of external signs of puberty onset. From PND 34, the vaginal opening (female) and preputial separation (male) of the pups (n = 10 per group) was noticed every day. The day that a complete preputial separation and vaginal opening was seen was considered as the day of puberty onset for that animal, and the average day of puberty onset for each group was attained and described.

Determination of estrous cyclicity. For the measurement of estrous cyclicity of the female rats, the vaginal smear from PND 60 to PND 70 was collected and observed under a microscope for 10 consecutive days. The uniformity of the estrous cycle was attained and described.

Daily sperm production (DSP). Frozen testicular tissues were thawed at room temperature; parenchyma was weighed after removing tunica albuginea. Testicular homogenate was prepared by solution (5 mL) containing sodium chloride (0.9%) and Triton X-100 (0.5%) using a rotor-stator homogenizer (IKA-Werke, Staufen, Germany) for 30 s. After that, the homogenate was diluted five times, and the sample was taken on Neubauer chamber and 19th-stage spermatids were counted under a light microscope at 40× magnification. At least three readings were taken for average number of spermatids calculations. To obtain the number of spermatids, these values were used and divided by 6.3 (number of days the spermatids remain in seminiferous epithelium) to determine DSP.

Daily sperm production(DSP) = $Y/6.3$.

Histopathology. Testis and ovaries collected from the experiment were fixed in 10% formalin for 48 h. Next to fixation, dehydration was carried out at room temperature in the ascending grades of alcohol, and tissues were shifted to xylene. After clearing, tissues were embedded in paraffin wax, and 5- to 7-μm-thick sections were cut using microtome (Thermo, Shandon Finesse 325, Cambridge, UK). Sections were transferred to albumenized slides that were preheated at 37°C. For complete stretching of tissues and removal of the bubbles from glass slides, slides were placed overnight in incubator at 58 C. Then, tissues were rehydrated in descending grades of alcohol, stained with hematoxylin–eosin stain, and covered with cover slip. Prepared slides were observed under Leica microscope (New York City, New York, USA) equipped with digital camera (Canon, Japan).

Hormonal analysis. Plasma levels of T were determined by enzyme-linked immunosorbent assay (ELISA) kit (Cat # BC-1115, Bio Check Inc., California, USA) based on the principle of competitive binding between T in the specimen and T-horseradish peroxidase (HRP) conjugate for a constant amount of rabbit anti-T. The FSH (RD-FSH-Ra, Reddot Biotech Inc., Kelowna, British Columbia Canada) ELISA is principally based on the competitive inhibition binding reaction between biotin-labeled FSH and FSH in the plasma test samples, with the FSH-specific antibody. LH (RD-LH-Ra, Reddot Biotech Inc. Kelowna, British Columbia, Canada) ELISA is principally based on the competitive inhibition binding reaction between biotin-labeled LH and LH in the plasma test samples, with the LH-specific antibody. Estrogen (ELISA) kits (Cat # ES-180S) are based on the principle of competitive binding between estrogen in the test specimen and estrogen-HRP conjugate for a constant amount of rabbit anti-estradiol. Progesterone concentration was quantitatively determined in plasma through ELISA kit (Cat # PG-129S), which was based on the principle of competitive binding between progesterone in the test specimen and progesterone-HRP conjugate for a constant amount of rabbit anti-progesterone. The ELISA kits were validated by verifying the slope of the curve obtained by serial dilutions (0%, 20%, 40%, 60%, and 80%) of the sample with ELISA buffer and the curve created with kit standards, T (slope (m) = 0.0697, r^2 = 0.988), FSH (m = 0.039, r^2 = 0.974), LH (m = 0.0241, r^2 = 0.96), estrogen (m = 0.066, r^2 = 0.966), and progesterone (m = 0.021, r^2 = 0.9824) indicating the positive linear relationship.

Statistical analysis

The results are stated as mean ± SD. Statistical analysis was done using lme4[33] and easyanova[34] package of R 3.2.5.[35] Using Shapiro–Wilks's, Levene's, and Tukey's 1 − df test, we tested the statement of normality, homogeneity of variances, and additivity of the model. The effects of different treatments were analyzed by analysis of variance ea1 command of R with completely randomized design, followed by post hoc Tukey's honest significant difference (HSD), while the data of indices, F0 head tilt, foot spray, parental F0 reproductive parameters (no. (%) of plug/sperm-positive females, no. (%) of pregnant females, no. of live litters on PND 0, no. of live litters on PND 4, etc.), and percentages were compared using χ^2 test. The values of $p < 0.05$ were considered statistically significant.

Results

Results of parental F0 weight gain after furan exposure

The significant decrease in bw of prebreed male (p = 0.02) 5 mg kg^{-1} and (p = 0.001) 10 mg kg^{-1} and prebreed female (p = 0.1) 5 mg kg^{-1} and (p = 0.03) 10 mg kg^{-1} was observed in the furan-treated groups when compared with the control (nontreated) group. Similarly, the F0 females showed significant decrease in bw during gestational period in highest dose (p = 0.05) 10 mg kg^{-1} furan-treated groups (Table 1). However, the bw of F0 females significantly increased during lactation period in 5 mg kg^{-1} (p = 0.05) and 10 mg kg^{-1} (p = 0.02) furan-treated groups when compared to the control group (Table 1).

Table 1. Effects of oral furan (0, 1, 2.5, 5, and 10 mg kg^{-1}) exposure on F0 parental body weight gains.[a]

	Furan (mg kg^{-1})					
F0	Control	1	2.5	5	10	Statistics
Prebreed (*n*)	30	30	30	30	30	
Males (g)	219.36 ± 9.19[b]	206.5 ± 11.67[bc]	198.83 ± 12.3[bd]	180.06 ± 6.33[cd]	169.76 ± 5.96[d]	$p = 0.001$, $F = 4.53$
Females (g)	199.16 ± 6.75[b]	188.96 ± 6.57[bc]	191.5 ± 9.14[bc]	176.93 ± 4.54[bc]	172.56 ± 5.63[c]	$p = 0.03$, $F = 2.65$
Gestation (*n*)	23	21	24	20	17	
Females (g)	105.13 ± 7.24[b]	97.26 ± 7.53[b]	95.73 ± 9.88[b]	81.13 ± 7.32[bc]	67.46 ± 14.09[c]	$p = 0.05$, $F = 2.36$
Lactation (*n*)	23	21	22	18	13	
Females (g)	19.75 ± 2.64[c]	23.68 ± 4.50[bc]	20.53 ± 2.46[bc]	28.25 ± 3.64[bc]	33.93 ± 4.41[b]	$p = 0.02$, $F = 2.93$

SEM: standard error of the mean.
[a]*p* and *F* values in the rows from analysis of variance with complete randomized designs followed by Tukey's post hoc show a pairwise comparison of control with furan-treated groups. Mean with different superscripts are significantly different ($p < 0.05$) in the rows compared to control with other treated groups. Values are presented as mean ± SEM.
[b]Significant at $p < 0.05$ versus control.
[c]Significant at $p < 0.001$ versus control.
[d]Significant at $p < 0.01$ versus control.

Table 2. Mean (±SD) results of effects of parental F0 head tilt and foot spray after furan exposure orally (0, 1, 2.5, 5, and 10 mg kg^{-1}).[a]

	Furan (mg kg^{-1})					
	Control	1	2.5	5	10	Statistics
F0 males						
No. of animals examined	30	30	30	30	30	
No. of animals (%) with head tilt	3 (10)	2 (6.66)	5 (16.66)	11 (33.33)	13 (43.33)	$p = 0.04$
No. of animals (%) with foot splay	2 (6.66)	2 (6.66)	5 (16.66)	8 (26.66)	11 (36.66)	$p = 0.02$
F0 females						
No. of animals examined	30	30	30	30	30	
No. of animals (%) with head tilt	3 (10)	2 (6.66)	1 (3.33)	6 (20)	7 (23.33)	$p = 0.1$
No. of animals (%) with foot splay	2 (6.66)	0 (0)	3 (10)	7 (23.33)	9 (30)	$p = 0.05$

[a]These data are presented as the number (%) of the 30 animals per specified dose group per sex, which exhibited the finding(s) at least once from weaning to demise. Mean with different superscripts are significantly different ($p < 0.05$) in the rows compared to control with other-treated groups using χ^2 test.

Results of parental F0 head tilt and foot splay after furan exposure

After the observation of physical parameters, a significant difference was observed in head tilt and foot splay of F0 males of furan-treated groups when compared with control group. Similarly, in F0 females, only significant difference was also observed in foot splay (Table 2).

Results of parental F0 reproductive parameters after furan exposure

The reproductive parameters of F0 parental rats (to delivered F1 offspring) are presented in Table 3. The percentage of plug/sperm-positive females ($p = 0.97$), age of pregnant females (66.6% and 56.6%, respectively), and gestational length ($p = 0.87$) were nonsignificantly changed among all furan-treated groups as compared to control group (Table 3). Additionally, the number of live pups/litters was reduced significantly in 10 mg kg^{-1} groups when compared with the control group ($p = 0.001$, $F = 4.81$; Table 3).

Results of F1 generation results during lactation period

In F1 generation, total number of pups born and total number of live pups on PND 0, PND 4, PND 7, PND 14, PND 21, and PND 28 were significantly ($p < 0.001$) reduced in 10 mg kg^{-1} furan-treated group. There was no difference observed in sex ratio and survival of F1 pups during lactation period. The litter

Table 3. Results of effects of parental F0 reproductive parameters after furan exposure orally (0, 1, 2.5, 5, and 10 mg kg^{-1}).[a]

	Furan (mg kg^{-1})					
	Control	1	2.5	5	10	Statistics
No. of F0 pairs at mating (*n*)	30	30	30	30	30	
No. (%) of plug/sperm-positive females	27 (90)	25 (83.33)	29 (96.33)	25 (83.33)	25 (83.33)	$p = 0.97$
No. (%) of pregnant females	23 (83.33)	21 (76.66)	24 (80)	20 (66.66)	17 (56.66)	$p = 0.83$
No. of live pups/litter (PND 0)	9.90 ± 2.11[b]	9.81 ± 2.09[b]	9.45 ± 2.05[b]	6.54 ± 1.39[bc]	5.72 ± 1.22[c]	$p = 0.001$, $F = 4.81$
No. of live litters on PND 0	23	21	22	19	15	$p = 0.73$
No. of live litters on PND 4	23	21	22	18	13	$p = 0.49$
Gestational length (*d*)	22.1 ± 4.71	22.0 ± 4.70	22.2 ± 4.73	22.4 ± 4.77	22.5 ± 4.79	$p = 0.87$, $F = 0.30$
Index (%)						
Fecundity index (%)	85.18	84	80	80	68	$p = 0.67$
Fertility index (%)	76.66	70	80	66.66	56.66	$p = 0.31$
Gestational index (%)	100	100	100	94.73	86.66	$p = 0.84$

PND: postnatal day; SD: standard deviation.

[a]*p* and *F* values in the rows of gestational length and no. of live pups/litter (PND 0) from analysis of variance with complete randomized designs followed by Tukey's post hoc show a pairwise comparison of control with furan-treated groups. Data of number (%) and index were compared using χ^2 test. Mean with different superscripts are significantly different ($p < 0.05$) in the rows compared to control with other treated groups. Values are presented as mean ± SD.

[b]Significant at $p < 0.05$ versus control.

[c]Significant at $p < 0.001$ versus control.

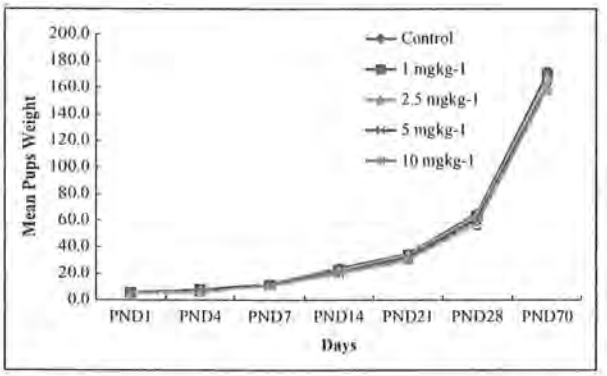

Figure 1. Mean body weights of F1 litters from PND 1 to PND 70. Data are presented as mean ± SD. ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with parently furan (0, 1, 2.5, 5, and 10 mg kg^{-1}) exposure groups in F1 generation. ANOVA: analysis of variance; PND: postnatal day.

size of F0 high-dose dams was reduced significantly ($p < 0.001$) during the lactation periods. Similarly, the bw of all the pups on PND 4 was reduced significantly ($p < 0.001$) in 10 mg kg^{-1} furan-treated group (Figures 1-3). AGD was nonsignificantly affected by treatment in both male and female pups. Males usually have zero nipples, but sometimes a few nipples could be observed. The exposure of parents to furan did not affect the NR in F1 males ($p = 0.07$, $F = 2.28$; Table 4).

Results of F1 generation at PND 70

From PND 30 onward, all the male and female pups were analyzed daily for preputial skin separation and vaginal opening. The day when the preputial skin was separated and the vaginal opening appeared was consider as day 1 of puberty. The analysis of external signs of puberty onset showed that parental furan exposure did not affect the pup's puberty (Table 5). On PND 70, male and female rats were dissected. Results of male rats indicate that the testicular weight was significantly ($p = 0.03$) decreased in the high-dose treated group (10 mg kg^{-1}), while there were no significant change observed in weight of prostate, seminal vesicle, and epididymis in dose-dependent manner compared with control group. The DSP was also significantly decreased in 5 mg kg^{-1} ($p = 0.04$) and 10 mg kg^{-1} ($p = 0.01$) treated group. Similarly, in female rats at PND 70, no significant change was observed in uterus weight, while a significant ($p = 0.02$) decrease in paired ovary weight was observed in 10 mg kg^{-1} furan-treated groups. The estrous cycle

Table 4. Results of F1 litters parameters during lactation.[a]

	Furan (mg kg^{-1})					
	Control	1	2.5	5	10	Statistics
No. of litters delivered	23	21	22	19	15	$p = 0.77$
No. of total pups born	189	183	181	104	94	$p < 0.001$
No. of live pups (PND 0)	184	176	174	102	92	$p < 0.001$
No. of live pups (PND 4)	184	176	174	100	89	$p < 0.001$
No. of live pups (PND 7)	184	176	174	100	89	$p < 0.001$
No. of live pups (PND 14)	184	176	174	100	89	$p < 0.001$
No. of live pups (PND 21)	184	176	174	100	89	$p < 0.001$
No. of live pups (PND 28)	181	174	169	99	87	$p < 0.001$
Sex ratio (% males, PND 0)	50.10 ± 7.56	44.54 ± 5.76	47.12 ± 8.78	46.73 ± 12.84	48.61 ± 15.85	$p = 0.95$, $F = 0.16$
Live litters size (PND0)	9.57 ± 0.16[b]	9.46 ± 0.22[b]	9.21 ± 0.33[b]	6.96 ± 0.64[c]	5.87 ± 0.35[c]	$p < 0.001$, $F = 19.67$
AGD in males	3.7 ± 0.22	3.63 ± 0.24	3.54 ± 0.24	3.53 ± 0.11	3.51 ± 0.12	$p = 0.95$, $F = 0.16$
AGD in females	2.41 ± 0.08	2.36 ± 0.09	2.39 ± 0.07	2.35 ± 0.08	2.36 ± 0.09	$p = 0.98$, $F = 0.08$
NR in male	0.26 ± 0.02	0.22 ± 0.02	0.21 ± 0.01	0.19 ± 0.02	0.18 ± 0.01	$p = 0.07$, $F = 2.28$
Indices (%)						
Live birth index	100	98.90 ± 4.23	98.26 ± 6.72	97.5 ± 2.50	97.89 ± 5.96	$p = 0.99$, $F = 0.04$
4-Day survival	97.48 ± 2.51	96.70 ± 3.00	98.12 ± 1.83	98.04 ± 3.93	96.53 ± 2.84	$p = 0.98$, $F = 0.08$
7-Day survival	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	$p = 0.91$, $F = 0.23$
14-Day survival	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	$p = 0.91$, $F = 0.23$
21-Day survival	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	$p = 0.91$, $F = 0.23$
28-Day survival	98.55 ± 1.37	99.24 ± 1.03	97.32 ± 2.31	99.33 ± 0.95	97.90 ± 2.25	$p = 0.92$, $F = 0.22$
Lactation index	98.55 ± 1.37	99.24 ± 1.03	97.32 ± 2.31	99.33 ± 0.95	97.90 ± 2.25	$p = 0.92$, $F = 0.22$

NR: nipple retention; PND: postnatal day; AGD: anogenital distance; SD: standard deviation.
[a]p and F values in the rows from analysis of variance with complete randomized designs followed by Tukey's post hoc show a pairwise comparison of control with furan-treated groups. Mean with different superscripts are significantly different ($p < 0.05$) in the rows compared to control with other treated groups. Values are presented as mean ± SD.
[b]Significant at $p < 0.05$ versus control.
[c]Significant at $p < 0.001$ versus control.

length of female rats was also significantly ($p < 0.01$) increased in 5 mg kg^{-1} and 10 mg kg^{-1} treated group compared to control group (Table 5).

Results of F1 generation histopathology of testis and ovary at PND 70

On PND 70, parental exposure to different concentrations of furan exhibited some obvious changes in the testis histology of F1 generation. The lumen diameter was increased in higher dose treated groups. Changes were also found in tubular diameter and epithelial height but were not statistically different in the furan-exposed groups when compared with the control group (Table 6; Figure 4). Histological analysis of ovary revealed the presence of different types of ovarian follicles, that is, primordial follicle, primary follicle, secondary follicle, antral follicle, atretic follicle, cystic follicle, and corpus luteum (Table 6). Many minor differences were observed in follicles of female ovary. However, the number of cystic follicles was prominently observed in 10 mg kg^{-1} treated group (Table 6; Figure 5).

Results of F1 generation hormonal analysis at PND 70

There were no statistically significant difference observed in FSH and LH concentration in 10 mg kg^{-1} furan-treated male groups compared with the control group, while the plasma T level was significantly ($p = 0.03$) reduced in 10 mg kg^{-1} treated group (Table 7). Similarly, in female rats, plasma concentration of estrogen was reduced significantly ($p = 0.05$), while the levels of progesterone, LH, and FSH were not statistically different in 10 mg kg^{-1} treated groups when compared with control animals (Table 7).

Discussion

After fertilization, complex physiological and endocrine mechanisms are involved, which control the embryonic development. Any alteration in these mechanisms can cause abnormal growth and functional changes in the life of an adult organism. It has been previously observed that, when parents were exposed to environmental toxicants, it may cause

Table 5. Results of F1 generation at PND 70.[a]

	Furan (mg kg^{-1})					
	Control	1	2.5	5	10	Statistics
Mean pup weight (g), PND 70 (*n*)	170.28 ± 3.32	169.57 ± 5.12	166.14 ± 3.40	160.42 ± 4.80	158.64 ± 3.42	$p = 0.16$, $F = 1.66$
Mean male pup weight (g), PND 70 (*n*)	171.60 ± 3.17	170.28 ± 5.09	167.50 ± 2.20	160.42 ± 3.27	160.78 ± 3.08	$p = 0.07$, $F = 2.20$
Mean female pup weight (g), PND 70 (*n*)	169.49 ± 3.33	167.64 ± 5.39	164 ± 2.21	161.14 ± 2.81	161.50 ± 3.56	$p = 0.39$, $F = 1.02$
Paired testis weight	2.97 ± 0.08[b]	2.82 ± 0.17[bc]	2.63 ± 0.11[bc]	2.46 ± 0.17[bc]	2.20 ± 0.20[c]	$p = 0.03$, $F = 2.82$
Epididymis weight	1.24 ± 0.09	1.12 ± 0.04	1.01 ± 0.02	1.05 ± 0.01	1.00 ± 0.02	$p = 0.37$, $F = 1.08$
Prostrate	1.20 ± 0.09	1.16 ± 0.02	1.02 ± 0.02	1.23 ± 0.07	1.19 ± 0.09	$p = 0.37$, $F = 1.07$
Seminal vesicles	0.75 ± 0.08	0.75 ± 0.08	0.76 ± 0.09	0.68 ± 0.06	0.71 ± 0.07	$p = 0.97$, $F = 0.12$
DSP $\times 10^6$	87.6 ± 2.48[b]	81.2 ± 4.18[bc]	75 ± 3.37[bc]	71.4 ± 4.36[c]	68.8 ± 2.93[c]	$p = 0.01$, $F = 4.03$
Paired ovary weight	127.9 ± 7.48[b]	127.3 ± 5.99[b]	125.7 ± 2.87[b]	114.4 ± 3.33[bc]	106.8.4 ± 1.74[c]	$p = 0.02$, $F = 2.95$
Uteri weight	0.97 ± 0.01	0.92 ± 0.05	0.94 ± 0.02	0.88 ± 0.02	0.84 ± 0.03	$p = 0.21$, $F = 1.50$
Estrous cycle length	4.13 ± 0.06[b]	4.15 ± 0.06[bc]	4.22 ± 0.08[bc]	4.42 ± 0.11[c]	4.56 ± 0.12[c]	$p = 0.008$, $F = 3.92$
Vaginal opening	32.03 ± 0.14	31.99 ± 0.13	32.14 ± 0.15	32.18 ± 0.12	32.27 ± 0.08	$p = 0.54$, $F = 0.77$
Preputial separation	43.05 ± 0.23	43.11 ± 0.24	43.09 ± 0.32	43.18 ± 0.25	43.27 ± 0.23	$p = 0.97$, $F = 0.11$

DSP: daily sperm production; PND: postnatal day; SD: standard deviation.
[a]*p* and *F* values in the rows from analysis of variance with complete randomized designs followed by Tukey's post hoc show a pairwise comparison of control with furan-treated groups. Mean with different superscripts are significantly different ($p < 0.05$) in the rows compared to control with other treated groups. Values are presented as mean ± SD.
[b]Significant at $p < 0.05$ versus control.
[c]Significant at $p < 0.001$ versus control.

Table 6. Results of F1 generation histopathology at PND 70.[a]

	Furan (mg kg^{-1})					
	Control	1	2.5	5	10	Statistics
Male testis						
Tubular diameter	213 ± 10.55	205 ± 13.9	194 ± 9.01	182 ± 8.16	177 ± 8.58	$p = 0.11$, $F = 1.90$
Lumen diameter	30 ± 2.00[b]	32 ± 2.84[b]	32 ± 1.89[bc]	37 ± 2.15[c]	39 ± 2.88[c]	$p = 0.04$, $F = 1.53$
Epithelial heights	64 ± 4.04	65 ± 3.71	69 ± 3.51	70 ± 2.87	71 ± 4.23	$p = 0.64$, $F = 0.62$
Female ovary						
Primordial follicle	368 ± 12.2	350 ± 11.3	335 ± 10.7	330 ± 9.49	330 ± 10.1	$p = 0.07$, $F = 2.27$
Primary follicle	191 ± 9.18	185 ± 10.2	177 ± 10.6	168 ± 6.62	166 ± 8.75	$p = 0.24$, $F = 1.39$
Secondary follicle	68.8 ± 3.28	67.3 ± 2.05	64.02 ± 2.49	61.3 ± 2.08	60.02 ± 2.52	$p = 0.07$, $F = 2.22$
Cystic follicle	1.91 ± 0.21[c]	1.96 ± 0.16[bc]	2.16 ± 0.20[bc]	2.41 ± 0.20[b]	2.69 ± 0.20[b]	$p = 0.05$, $F = 2.40$
Atretic follicle	2.53 ± 0.07	2.54 ± 0.03	2.88 ± 0.01	3.04 ± 0.01	3.11 ± 0.18	$p = 0.59$, $F = 0.70$
Corpus luteum	10.79 ± 0.45	10.72 ± 0.75	9.83 ± 1.00	10.20 ± 0.20	9.51 ± 0.36	$p = 0.53$, $F = 0.79$
POFs	2.86 ± 0.05	2.71 ± 0.05	2.62 ± 0.09	2.33 ± 0.06	2.26 ± 0.07	$p = 0.15$, $F = 1.74$

POF: preovulatory follicle; PND: postnatal day; SD: standard deviation.
[a]*p* and *F* values in the rows from analysis of variance with complete randomized designs followed by Tukey's post hoc show a pairwise comparison of control with furan-treated groups. Mean with different superscripts are significantly different ($p < 0.05$) in the rows compared to control with other treated groups. Values are presented as mean ± SD.
[b]Significant at $p < 0.001$ versus control.
[c]Significant at $p < 0.05$ versus control.

developmental, behavioral, and hormonal changes in the body of offspring.[23,30,36] In the present study, the potential effects of furan on rats were evaluated in an EOGRTS and demonstrated that long-term exposure of furan to parents resulted in parental F0 toxicity and F1 generation toxicity in both sexes at higher doses (5 mg kg^{-1} and 10 mg kg^{-1}). Normal development of the male and female reproductive systems depends upon the proper functioning of testis, ovaries, pituitary, and hypothalamus. The hormonal secretions

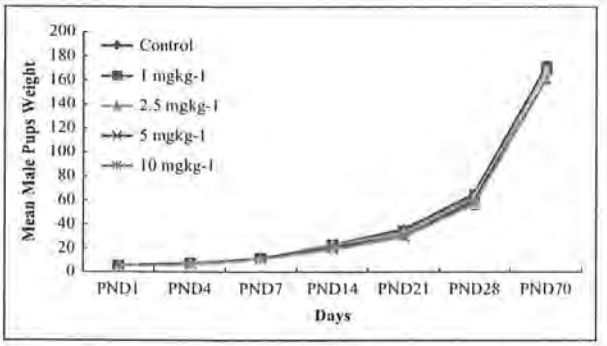

Figure 2. Male body weights of F1 litters from PND 1 to PND 70. Data are presented as mean ± SD. ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with parently furan (0, 1, 2.5, 5, and 10 mg kg^{-1}) exposure groups in F1 generation. ANOVA: analysis of variance; PND: postnatal day; SD: standard deviation.

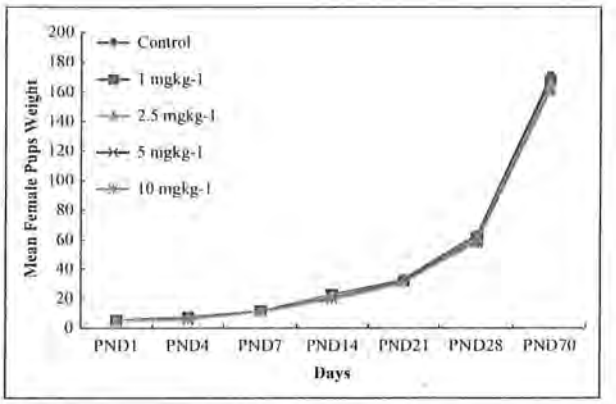

Figure 3. Female body weights of F1 litters from PND 1 to PND 70. Data are presented as mean ± SD. ANOVA with complete randomized designs followed by Tukey's post hoc shows a pairwise comparison of control with parently furan (0, 1, 2.5, 5, and 10 mg kg^{-1}) exposure groups in F1 generation. ANOVA: analysis of variance; PND: postnatal day; SD: standard deviation.

from pituitary and gonads are necessary for the development of reproductive systems.

Endocrine regulation involves the secretion of gonadotropin-releasing hormone from hypothalamus that further stimulates the secretion of LH and FSH from anterior pituitary, all these hormones are necessary for the normal functions of reproduction (spermatogenesis and folliculogenesis).[37–39] It has been previously observed that exposure to some endocrine disrupting chemicals (EDCs) can lead to disturbance of the several normal body functions. In rodents during the development period, exposure to EDCs at higher concentrations disturbed the reproductive functions in adulthood.[40] Similarly, human exposure to EDCs brings changes in reproductive functions, and genital abnormalities are evident in last half century.[41,42] Exposure to environmental toxicants, for example, acrylamide, bisphenol, tetrabromobisphenol A, and imidacloprid, brought variations in growth and endocrine functions and thus affected F1 and F2 generations.[23,30,36,43]

It has been previously reported that exposure of furan caused reproductive toxicity in several animal studies.[5,16] Guo et al. reported that young men prenatally exposed to dioxins and furan have a high incidence of abnormal sperm morphology, motility, and reduced ability to penetrate hamster ovary.[22] Furan was also investigated to induce chromosomal aberrations and sister chromatid exchanges in Chinese hamster ovary cells and Chinese hamster V79-derived cell line.[13,44] Previously, it has been observed that different concentrations of furan induced toxic effects on the endocrine systems of animals and humans.[5,12,16]

In current investigation, a significant change in F0 parental weight gain of males (prebreed period) and female (prebreed period, gestation, and lactation) was observed after exposure with high doses of 5 mg kg^{-1} and 10 mg kg^{-1} of furan. Similar findings previously reported that reduction in weight gain of animals was observed when exposed to environmental toxicants such as acrylamide, furan, and bisphenol.[6,23,30] The toxicity of furan at parental stage (F0) in current study is similar to the previous findings. In animals, EDCs has been widely studied, and different physical parameters have been estimated and standardized to confirm the toxicological effects of EDCs on development of different organs by physical examination. Study of these parameters reveals the state of development of organs within the body. Subchronic furan and acrylamide exposure resulted in reduced seminal vesicle and testicular weight, along with reduced serum level of T, LH, FSH, and prolactin.[6,19,45] The bw is the most sensitive indicator of adverse effect of xenobiotics. It is notable that observing the bw is important for interpretation of reproductive effects of toxins.[46] The results of the current study showed the decrease in prebreed bw gain of F0 (parents). Explanation of the reduced bw may be due to growth and protein deficiencies because of malnutrition during development.[47,48] It may also be due to excessive breakdown of tissue and plasma protein.[49] In current

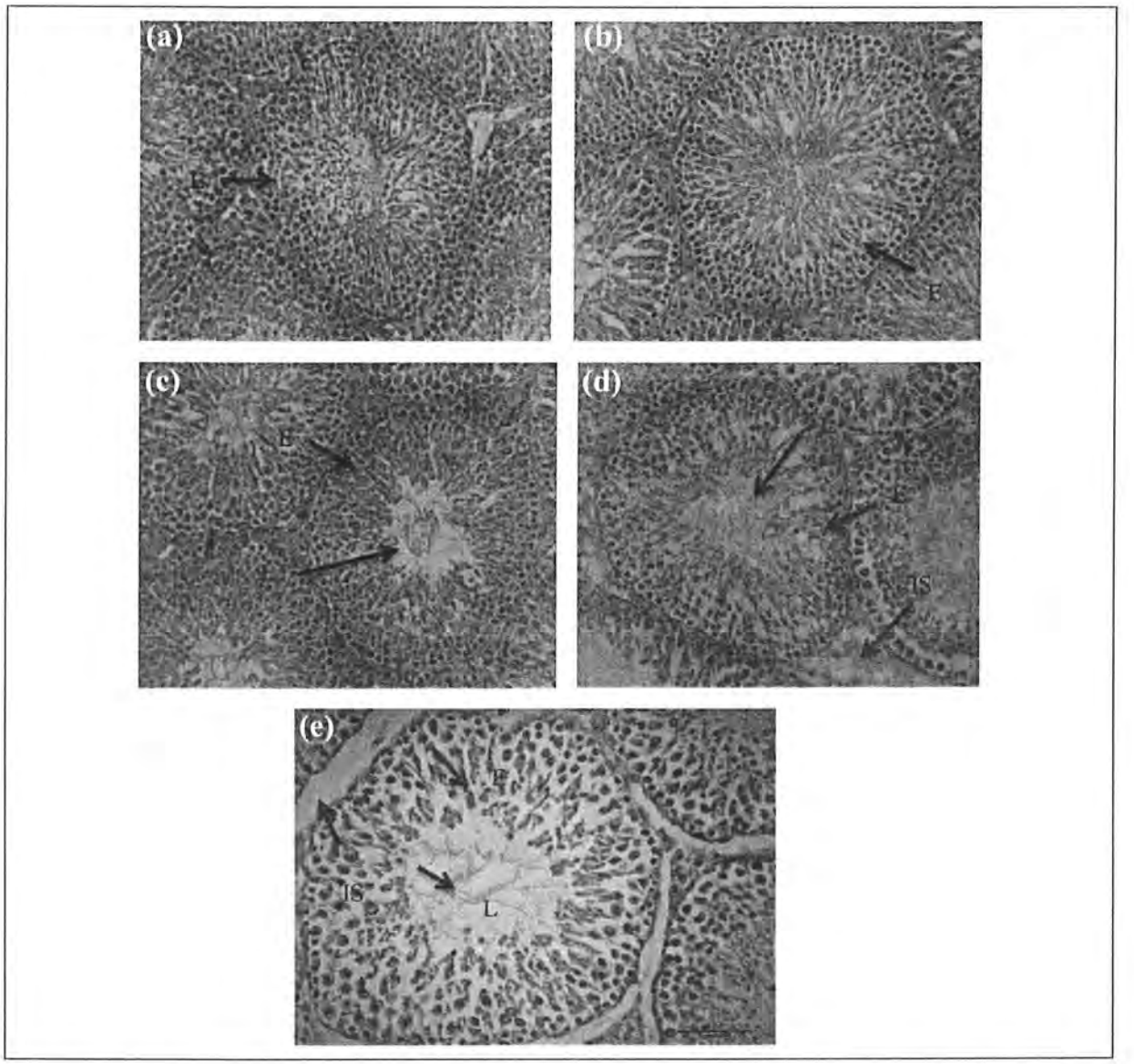

Figure 4. Photomicrograph (×40) of cross section of seminiferous tubules of male rats in F1 generation, with both parents receiving doses of furan: (a) Control group shows regularly arranged tubules with thick epithelium lumen filled with spermatids; (b) 1 mg kg^{-1} group shows normal epithelial height, lumen filled with mature spermatozoa; (c) 2.5 mg kg^{-1} group shows normal morphology, lumen filled with immature spermatozoa; (d) 5 mg kg^{-1} group shows increased lumen diameter and interstitial spaces; (e) 10 mg kg^{-1} group shows thin degenerated epithelium with empty lumen and interstitial spaces. L: lumen; E: epithelium; IS: interstitial spaces.

study, weight gain in females during gestation period was decreased, while increase in bw gain was noticed during lactating period. This might be due to increased food consumption during gestation and lactation period. Metabolism of females increases during gestation and lactation period, and females gain additional calories during this period due to fast metabolism.[50] In our study, reduction in gestational weight gain may be due to the reason that lactation makes greater demand of mother's body than pregnancy does.[43] Another possible reason is low number of implantation sites during pregnancy.

In this current study, head tilt and foot splay were also observed in F0 parents after exposure with various doses of furan. This is related to the neurotoxic

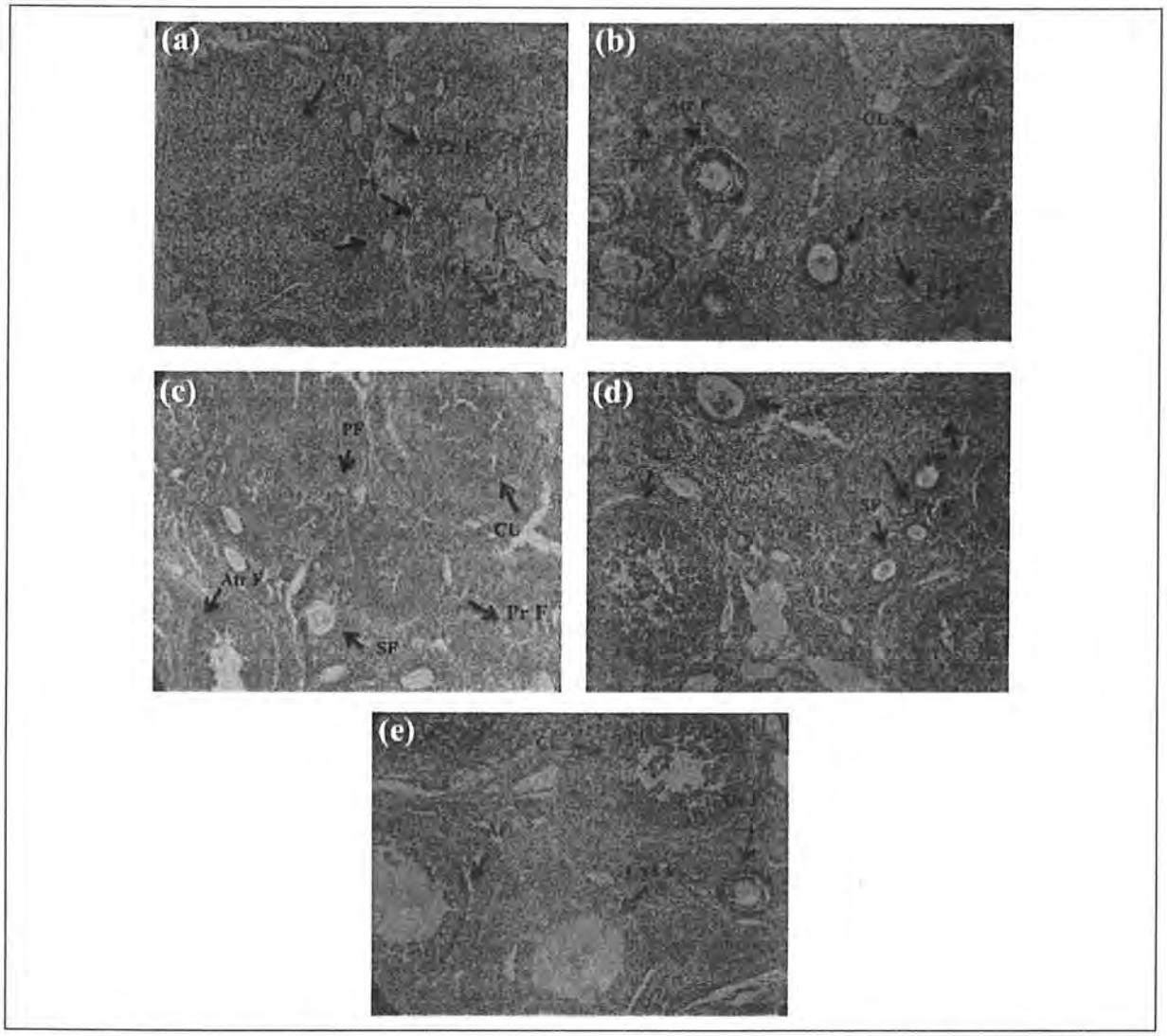

Figure 5. Photomicrograph (40×) showing different stages of folliculogenesis in ovarian tissues of rats in F1 generation, with both parents receiving doses of furan: (a) control group shows all the stages of folliculogenesis; (b) 1 mg kg^{-1} group shows no significant effect on number of follicles as compared to control group; (c) 2.5 mg kg^{-1} group represents no significant difference except a slight decrease in number of ovarian follicles; (d) 5 mg kg^{-1} group shows reduction in number of ovarian follicles; (e) 10 mg kg^{-1} group indicates large number of Cys Fs in ovary. Pr F: primordial follicle; PF: primary follicle; SF: secondary follicle; AF: antral follicle; Atr F: atretic follicle; Cys F: cystic follicle; CL: corpus luteum.

effects of furan. Similar results were obtained previously in which acrylamide exposure caused head tilt and foot splay in high treatment groups, and results were related to the degeneration of brain monoaminergic system.[32] Spindle fiber proteins present in peripheral axons play an important role in anterograde axonal transport. It was reported that acrylamide interfered with these proteins and caused hind limb weakness like foot splay, reduce grip strength, and so on.[51] The effects observed in present study must be related to neurotoxic and reproductive toxic nature of furan.

In current study, reduction in number of pregnant F0 female was observed in higher dose treated groups. The possible reason for reduced pregnancy may be that furan generates ROS in sperm cells and causes sperm DNA damage.[12] Another reason is mounting behavior/libido failure.[23] Furan treatment might affect the rat's limbs grip, disturbing mounting behavior, so that they cannot penetrate sperm into the

Table 7. Results of F1 generation hormonal profile at PND 70.[a]

	Furan (mg kg^{-1})					
	Control	1	2.5	5	10	Statistics
Male						
Testosterone (ng mL^{-1})	6.86 ± 0.48[b]	6.12 ± 0.47[b]	6.00 ± 0.33[b]	5.34 ± 0.41[bc]	4.44 ± 0.43[c]	$p = 0.03$, $F = 2.77$
LH (ng mL^{-1})	2.33 ± 0.13	2.31 ± 0.13	2.30 ± 0.13	2.04 ± 0.17	1.80 ± 0.17	$p = 0.06$, $F = 2.34$
FSH (mIU mL^{-1})	1.23 ± 0.18	1.24 ± 0.07	1.21 ± 0.14	1.14 ± 0.08	1.04 ± 0.07	$p = 0.77$, $F = 0.45$
Female						
LH (ng mL^{-1})	2.69 ± 0.03	2.67 ± 0.02	2.63 ± 0.03	2.60 ± 0.07	2.53 ± 0.04	$p = 0.89$, $F = 0.27$
FSH (mIU mL^{-1})	4.03 ± 0.27	4.00 ± 0.21	3.77 ± 0.44	3.60 ± 0.36	3.10 ± 0.31	$p = 0.27$, $F = 1.30$
Estrogen	6.2 ± 0.42[b]	6 ± 1.21[b]	5.8 ± 0.51[bc]	4.9 ± 0.55[bc]	3.8 ± 0.53[c]	$p = 0.05$, $F = 2.49$
Progesterone	22.06 ± 1.31	20.66 ± 2.02	19.86 ± 2.29	19.66 ± 1.56	19.53 ± 1.77	$p = 0.85$, $F = 0.33$

PND: postnatal day; LH: luteinizing hormone; FSH: follicle-stimulating hormone; SD: standard deviation.
[a]p and F values in the rows from analysis of variance with complete randomized designs followed by Tukey's post hoc show a pairwise comparison of control with furan-treated groups. Mean with different superscripts are significantly different ($p < 0.05$) in the rows compared to control with other treated groups. Values are presented as mean ± SD.
[b]Significant at $p < 0.05$ versus control.
[c]Significant at $p < 0.001$ versus control.

uterus of females resulting in reduced pregnancy. Previously, it is reported that human exposure to furan revealed that furan affected lymphocytes and sperm cells by producing ROS.[12]

AGD development is related to the reproductive development. The distance between anus and genitalia of an organism reveals the degree of development of the reproductive system of the animal.[52] In current study, there was no significant difference observed in the male and female AGD and male NR in the furan-exposed group as compared to the control group. In the last 20 years, during critical period of sexual differentiation, the assessment of NR and AGD has been considered as important marker of altered androgen exposure.[53,54] These parameters are used to study the hazardous effects of toxins on reproductive toxicity in adulthood.[54,55] According to OECD protocols, these parameters are considered mandatory for testing of newly synthesized chemicals.[56]

In our study, we observed the reduced fertility rate/number of pups in F1 generation in high-dose treatment group (10 mg kg^{-1}). It is well known that the total number of live pups and litter size reduction is correlated with the reduction in implantation sites.[57] Similarly, chemical exposure suppresses the LH surge or delays preovulatory LH surge, which is correlated with delayed or blocked ovulation.[57,58] This continuous suppression of LH and delayed ovulation is associated with the significant reduction in litter size and number of implantation sites that affect number of live pups, fertility, and mating process. Although the AGD in males and females were not significantly affected, a mild decrease was observed. Reduced weight in pups was observed only in PND 0 and PND 4 when the pups depend on their mother for milk. This may be due to the furan exposure to the mother may cause detrimental effects in term of milk quality and quantity and may have potential toxic effects on the growth retardation of the offspring. No postnatal mortality was observed in F1 generation. Aforementioned data on the F0 rats are directly related with reported studies on acrylamide and furan reproductive toxicity.[5,6,16] However, one of the main purposes for EOGRTS is to provide the awareness about chemical toxicity in parents, offspring, and adulthood life stage.

It was noticed that puberty onset in males and females in F1 generation was not affected significantly in the current study. Similarly, no difference was observed in the bw gain on PND 70, but testicular and prostate weight were reduced dose dependently. Previous study has stated that prostrate and testicular weights depend on sex hormones as prostrate response to androgen antagonists is well established[59] and testicular dependency on androgen has also been shown.[60] Similarly, in this study, paired ovarian weight was also reduced in F1 generation females dose dependently. It means that exposure to furan during sensitive development period of life badly effects the accessory organ weight. Similarly, in an early study, decrease in ovarian weight was also recorded.[61]

The hormonal concentration, that is, T, LH, and FSH in males on PND 70, is slightly affected in F1 generation. In current finding, T and LH levels were

reduced in male rats because of the furan toxicity on reproductive system. DSP was also reduced in our current study because of low T concentration. T is essential for maintaining all functions of accessory sex glands,[16] and the lack of T disrupts spermatogenesis.[62] Similarly, female's estrogen level was reduced in high treatment group, but progesterone, LH, and FSH concentrations in adult animals were not affected. In addition, estrous cycle was disturbed with increase in estrous cycle length of F1 animals at higher doses. Our findings are supported by previous studies in which premenarcheal exposure of women with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was associated with longer menstrual cycles.[63] In mammalian reproduction, FSH is main hormone; it play a vital role in gonadal development, maturation at puberty, and gamete production during the fertile phase of life.[64] Estrogen and FSH concentration is important for female secondary sexual characteristics, development, and proliferation of the ovarian follicles, and it plays a significantly important role in women's health.[64,65] Significant changes in ovarian and testicular architecture were seen in F1 generation as examined by histological analysis. Spermatogenesis and folliculogenesis are dependent on endocrine regulation of sex hormones.[37,38,66] Results from the present study confirmed that parental furan exposure generated alterations in the hypothalamic pituitary testicular and ovarian axis that altered folliculogenesis and spermatogenesis in F1 generation. The parental furan exposure disturbed the levels of T in male rats and estrogen level in female rats in F1 generation. The testicular and ovarian weights were reduced in F1 generation at PND 70 with decreased DSP and disturbed estrous cyclicity in groups treated with high dose (10 mg kg^{-1}) of furan. Histopathological changes were also observed in testis and ovaries of F1 generation, whose parents were previously exposed with higher (10 mg kg^{-1}) doses of furan. Our results are in line with previous findings on furan exposure and highlight that parental exposure to furan can cause adverse effects on the F1 generation.

Conclusion

Our study demonstrates for the first time that extended one-generation exposure of rats to furan at different concentrations impairs their hypothalamic pituitary testicular and ovarian axis, which over long-term exposure might affect generations. As we predict an adverse effect of the food-based toxicant furan to mammals, long-term and generation studies that include the detection of altered folliculogenesis and spermatogenesis are warranted. The toxicological profile of furan presented in this study supports the identification of furan as an endocrine disruptor of concern for human health. To enable environmental risk assessment, more comprehensive molecular-based studies are recommended to further clarify the health hazardous effects of furan in living organisms.

Declaration of conflicting interests

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