Evaluation of Protective Effects of Vitamin C against Toxicity Induced by Furan in Adult Male Sprague Dawley rats

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By

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Dedication

Dedicated to my Parents, Brothers, sister, and my respectful supervisor who have given me the opportunity to study at the best institutions and supported me throughout my life.

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and the material contained in this thesis is original. I have not previously presented any part of this work elsewhere for any other degree.

CERTIFICATE

This dissertation "Evaluation of protective role of Vitamin C against furan induced toxicity in male Sprague Dawley rats" submitted by Mr. Asif Ullah, is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the degree of Master of Philosophy in zoology.

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List of Abbreviations

ANOVA	Analysis of Variance
CAT	Catalase
EDC	Endocrine disruptive chemicals
EFSA	European Food Safety Authority
EIA	Enzyme Immunoassay
ELISA	Enzyme linked immunosorbent assay
FDA	The Food and Drug Administration
Fecl2	Ferrous Chloride
H_2O_2	Hydrogen Peroxide
HPG axis	Hypothalamic-pituitary-Gonadal axis
HPT axis	Hypothalamic-pituitary-testicular axis
HRP	Horse reddish peroxidase
HRP	Horse Reddish Peroxidase
NADH	Nicotinamide dinucleotide

NTP	National Toxicology Program
POD	Peroxidase
ROS	Reactive oxygen species
SOD	Super oxide dismutase
T	Testosterone
TBARS	Thiobarbituric acid
Vit C	Vitamin C
WHO	World health organization

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Abstract

Furan is a heterocyclic organic molecule. Furan causes adverse effects on the vital organ of rodents such as the liver, kidney, pancreatic, and renal cells. In past studies, it is also reported that furan causes toxicity in the endocrine and reproductive system in male rats. The current study was designed to examine the protective effect of Vit C against toxicity induced by furan in male Sprague Dawley rats. The experiment was conducted for 28 days. Rats were randomly divided into four groups (n = 5). Group-I was control, received corn oil, and while Group II was treated with Furan 30 mg/kg orally, Group III was given 200 mg/kg orally, Group IV was given Vit C 200 mg/kg, and furan 30 mg/kg. Vitamin C significantly decrease the TBARS and ROS levels in all tissues exposed to furan as compared to the control group While POD, CAT and SOD were ($P<0.05$) significantly lowered in the furan treated group. The furan treatment group showed a significant reduction in testosterone concentration as compared to the to ist group. Furan-treated groups showed alterations in the normal morphological architecture of testis. Furan caused a reduction in the number of spermatids and disruption of tubules. A non-significant decrease in the organ mass index was also observed in furan treated group. Vitamin C was found to be an antioxidant role that reduced furan-induced toxicity. Vitamin c reduced alterations in oxidative stress, as it also restored the morphological alterations as compared to the furan treatment group. Vitamin C also restored the level of testosterone. It is concluded vitamin C is beneficial to furan-induced toxicity in male Sprague Dawley rat.

Introduction

There has been a lot of concern in recent decades regarding the negative impact of many environmental toxicants on human health and reproduction. Exposure to environmental toxicants, for example, is one of the leading causes of reproductive dysfunction in humans. Because of their capacity to modify animal pathophysiology, certain environmental chemicals/toxicants have been classified as endocrine-disrupting compounds (EDCs). EDC are chemicals that enter the body exogenously. EDCs cause changes in the activities of the endocrine system as well as unfavorable health consequences in the complete organism, its progeny, or the entire population (WHO/ IPCS, 2002).Some researchers have defined EDCs as "exogenous chemicals or a mixture of chemicals that delay with any aspect of hormone action (Soto *et al*., 2012). (Thomas Zoeller et al., 2012). Nearly 800 chemicals have been identified that are known to disrupt normal endocrine function (Bergman *et al*., 2013). These chemicals act as a risk for the development of health problems such as infertility, cancer, obesity, metabolic syndrome, and neurodevelopmental issues (López-rodríguez *et al*., 2021).

Dioxins, polychlorinated biphenyls (PCBs), insecticides, pesticides, herbicides, alkylphenols, fungicides, phthalates, phytoestrogens, and bisphenol A are among the substances that make up EDCs. Heavy metals such as lead, cadmium, arsenic, and mercury, as well as polybrominated biphenyls, pharmaceutical medicines, food-based acrylamide, and furan (Abel et al., 2018; Crisp et al., 1998; Lehmann *et* al., 2018a; Pandir, 2015b; Coster & Larebeke, 2012; Fromberg et al., 2014). Endocrine disruptive substances may be in the form of insecticides, environmental toxins, bisphenol A and phthalates. Environmental toxins such as polychlorinated biphenyls and dioxins have been shown to affect reproductive function and hormone signaling in both female and male reproductive organs (Brehm & Flaws, 2019).

EDC's method of action and ability is such that they bind to nuclear receptors (NRs) or endocrine (Coster & Larebeke, 2012; Soto *et al*., 2012). EDCs can activate and resemble the natural mechanisms of hormones such as estrogen, androgen, estrogen-related receptor, pregnane X, constitutive androstane, aryl hydrocarbon, thyroid hormone, retinoid X, and glucocorticoid receptor (Mnif *et al*., 2011). EDCs can disrupt endogenous hormone synthesis, transportation, metabolism, and excretion (Coster & Larebeke, 2012) (Gore *et al*., 2015). When EDCs interact with additional EDCs, they might create a more harmful cocktail effect (Balaguer *et al*., 2017).

Besides hypothalamus-pituitary gland-gonads, hypothalamus-pituitary gland-adrenal and hypothalamus-pituitary gland-thyroid (abbreviated as HPG, HPA and HPT respectively). EDCs act on every endocrine axis. However, EDCs are most likely to target HPG, HPA and HPT (Lauretta et al., 2019).The EDC causes harm to the central nervous system (CNS), hypothalamus, and pituitary (Kabir et al., 2015).The appropriate functioning of the HPA and HPG axes is critical for human survival. The interaction of EDCs with the HPA and HPG axis changes how they work. Developmental stages are also vulnerable because during the stage of organ development the effects become irreversible leaving impact of modification to hormonal profile when exposure of animals to EDCs (Richter *et al*., 2007; Sajjad *et al*., 2018).

EDC interaction weakens the body's ability to effectively regulate the endocrine system, resulting in negative developmental, reproductive, and neurological consequences (Barakat *et al*., 2019). EDCs have a capability to permanently modify the adult reproductive function when animals are directly exposed to endocrine disrupting chemical (Dickerson & Gore, 2007). EDCs can infect the human body in a variety of ways (Gore *et al*., 2015; Lauretta *et al*., 2019) . Exogenous substances can mistakenly disrupt this sophisticated communication mechanism, resulting in health consequences. Many animals as well as humans are directly or indirectly exposed to a countless range of EDCs throughout their lives as a result of using various consumer products, having interactions with work, natural resources, medications, military duty or may be due to

other circumstances (La Meril *et al*., 2020). Distinct research in animals and humans indicate the impacts of EDCs at various developmental stages(Lee, 2018). The most prevalent routes of EDC exposure on a regular basis through food, inhalation, clothing, furniture, toys, cosmetics, soil, dust, and plastic bottles, among other things (Barakat *et al*., 2019). According to recent research, EDC exposure during the formative stage not only harms the live creature but also has an impact on future generations (Lauretta *et al*., 2019). Moreover, EDCs like BPA disrupt reproduction in later generations, even if offspring were never physically exposed, defining such as transgenerational effects (Rahman *et al*., 2020).

Toxicants in food caused by heat:

The heating of foods imparts a variety of tantalizing sensory properties, including taste, flavour, and colour. However, several harmful chemicals, such as furan and acrylamide, are generated during the heating process and may provide a greater health risk to consumers. In the current period, naturally occurring toxicants mostly induced during heating the food such as chloropropanols, acrylamide and furan have gotten a lot of attention (Wenzl *et al*., 2007). Chloropropanols and furans have been found in food since the late 1970s, while acrylamide was discovered in food only a few years ago. Swedish. Researchers discovered acrylamide in fried meals such as French fries, snacks, cereals and cereal-based goods, cookies, and coffee in April 2000 (Stadler, 2012; Tareke *et al*., 2002). According to the research of International Agency for Cancer furan and acrylamide have been classified as human carcinogens (Vranová & Ciesarová, 2009). The (CFSAN, 2004) Center for food Safety and Applied Nutrition lists acrylamide and furan all of its safety food programs. Many environmental and heat-induced food toxicants, such as TCDD, arsenic, bisphenol, furan, chlorinated dioxins, polychlorinated biphenyl DDT, and acrylamide, have since been discovered in infant formulae and breast milk. (Lehmann *et al*., 2018) have been also reported of infant biscuits and follow-up formulae.(Mojska *et al*., 2012).

Furan

Furan (CH_4O_4) is a heterocyclic organic molecule (according to CAS No.110-00-9). Furan is a five-membered aromatic ring and contains one oxygen atom and four carbon atoms responsible for serving as an essential intermediary in the production of a variety of compounds (Zheng et al., 2019). Furan is easily soluble in more than 10% concentration of benzene, ethanol, acetone and ether and least soluble in water (NTP, 2014). Furan was firstly reported in food items by (Maga& Katz, 2009). The International Agency for Research on Cancer ("IARC Monogr. Eval. Carcinog. Risks to Humans," 2006) declared furan as a potentially hazardous chemical for humans as well as animals. The European Food Safety Authority (EFSA, 2004; NTP, 1993) classified furan as carcinogenic and genotoxic. In May 2004, the FDA released data on furan, and in 2004 and 2009, on the topic of contaminants in the food chain; a preliminary report on furan by the EFSA Scientific Panel was released (EFSA, 2010). They detected furan in a various type of foods and products. The foods such as canned, jarred, and baked foods, coffee, and baby food was most likely to be contaminated by furan (Javed *et* al., 2021). Furan is an environmental and food toxin that is commonly found in infant formula, canned foods, and junk foods, according to reports (Abel *et al*., 2018; Karacaoğlu & Selmanoğlu, 2010). Furan is also produced as a by-product of solid types of foods that have been exposed to high-energy radiation or extreme temperatures (Bakhiya & Appel, 2010a). It has been reported in the environment as a component of wood smoke, cigarette smoke, gasoline exhaust, and diesel engine exhaust gas (IARC, 1995). Following this, the European Food Safety Authority (EFSA) issued an appeal for more information on the presence of furan in foods (EFSA, 2006). The most essential food items that serve as precursor molecules for furan are fatty acids, carbohydrates, amino acids, and ascorbic acid (Crews & Castle, 2007).

Figure 01: Chemical structure of furan (Arisseto, 2016)

Occurrence of Furan

Furan, which is generated during thermal processing, is easily vaporized due to its low boiling point. However, because furan accumulates conveniently in jarred foods and canned foods, this creates problems. Furan levels in a wide range of items (roasted coffee, baby meals, bakery products, etc.) have been measured and range from undetectable to 11,000 µg/kg (Zheng *et al*., 2019). Highest levels of furans, up to 11,000 µg/kg are present in roasted coffee because roasting takes place at higher temperatures than most other thermal processes, resulting in induction of furan as well as other pollutants (Arisseto, 2016). Presence of furan in coffee was firstly noticed in 1938 and after a few years, scientists validated the induction of furan in foods (Merritt *et al*., 1963). Besides heat-induced furan found in food, the traces of furan were also evidenced in gases released by engine exhaust, wood smoke and cigarette smoke as well. Focusing on heat-induced furan in foods, it was also found in vegetables and canned fruits as well (Bakhiya & Appel, 2010b; Becalski *et al*., 2016; Liu & Tsai, 2010). Later, the presence of furan was reviewed and published after 2010 enclosing that the largest levels of furan are found in meals that have been heated and preserved in sealed containers for example, baby foods, baked beans, preserved meat and soups (Moro *et al*., 2012; Seok & Lee, 2020; Shen *et al*., 2016; Stadler, 2012). As the presence of furan was evidenced in baby foods, furan was evidenced in cereal products as 168 microgram per kilogram and mean value was calculated as 15 by EFSA (2011). According to Brazilian survey, biscuits and wafers, cream crackers, and breakfast cereals such as corn flakes have highest levels of

furan. While furan is least identified in cakes and loaf breads (Arisseto, 2016). Furan can be procured by thermal decomposition. Vit C and free sugars are present in fruits juices. Recently in 2007, the formation, occurrence and presence of furan in food was analyzed (Crews & Castle, 2007). It is also showed that furan concentrations, storage, time, and temperature may increase its production while roasting (Palmers *et al*., 2015).

Formation of Furan

According to (Maga, 1979) the principal source of furan in diet is carbohydrates when they thermally breakdown such as lactose, fructose and glucose which are general forms of sugar. According to FDA generation of furan in food is carried out by the use of vast number of amino acids, carbohydrates, and proteins (such as casein, cysteine, and alanine). Vitamins (such as thiamin, dehydroascorbic acid, and ascorbic acid) and Polyunsaturated fatty acids (PUFA) are also used for generation of furan in food (Perez & Yaylayan, 2004).

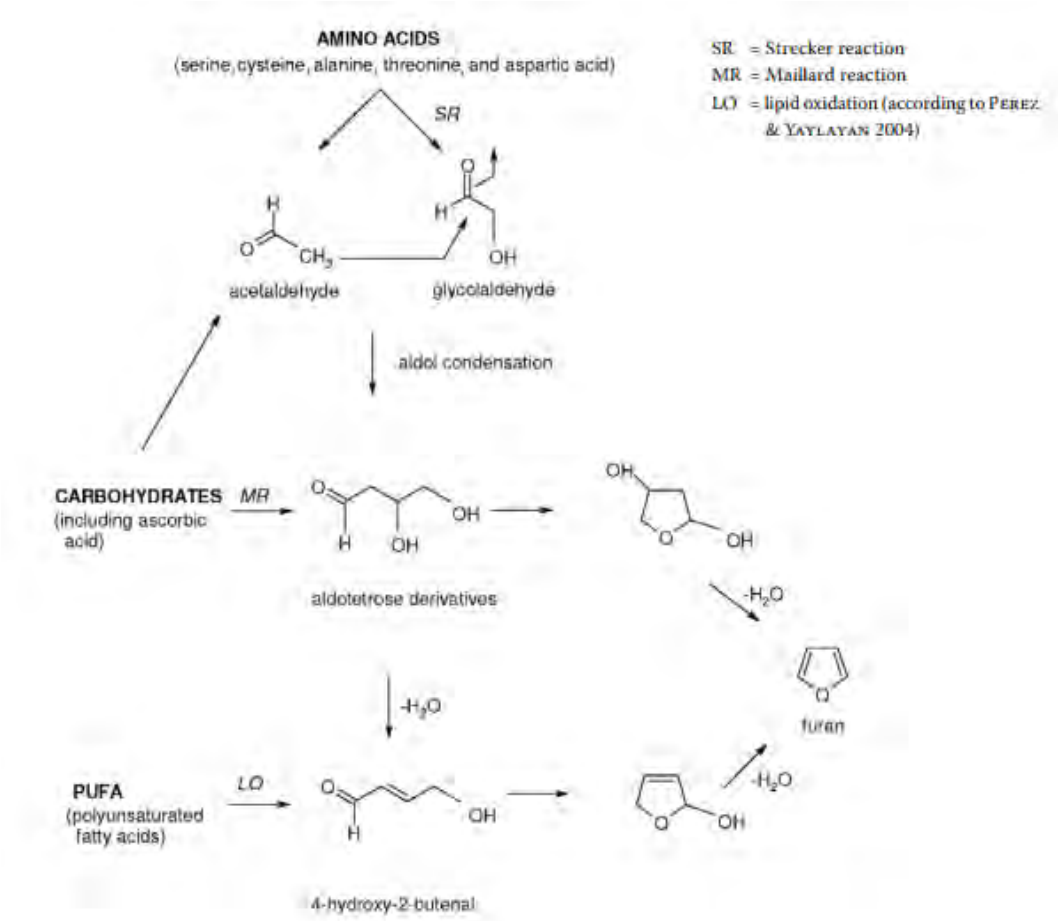


Figure 02: Different possible pathways for generation of furan from food precursor such as carbohydrates, amino acids and polyunsaturated fatty acids (Perez & Yaylayan, 2004)

Uses of Furan

The existence of furan in foods was recognized in the 1960s. Its manufacturing uses are mainly focused on synthetic purposes (Condurso *et* al., 2018). Furan is a very toxic chemical used in the formation of medicines, stabilizers, nylon, lacquers, and insecticides. Furan is also found in cigarette smoke, air pollutants and baby food (Moser *et al*., 2009). Industrially furan is used as intermediate compound for the synthesis of large number of organic compounds including drugs, lacquers, pesticides, and resins. For

the synthesis of thiophen, pyrrole, and tetrahydrofuran, furan is considered as an essential compound. In addition, furan is known as a by-product having radiations of powerful energy in food production (Bakhiya & Appel, 2010a).

Exposure of furan

Human food contains hundreds of structurally distinct chemical elements, the majority of which are of natural origin, as well as components that have been added for specific purposes such as colorants, nutrients, and flavors. European Food Safety Authority (EFSA) published the first report on the findings of furan level monitoring in 2009 (EFSA, 2009). Furthermore, Maximum furan contents were found by heat treatments, such as cereal items, soups, sauces infant food and meat products (EFSA, 2010). Human exposure to furan is high due to its widespread distribution in the environment (Santokh Gill et al., 2015). Human exposure to furan was determined based on feeding and the amount of furan present in various foods. Adults in developed nations are exposed to furan at a rate of 0.34 to 1.23 µg/kg body weight /day, or around 0.78 µg/kg bw/ day (EFSA, 2009). The average estimated exposure ranges up to 0.27 to 1.01 µg/kg bw/day for aged 3 to 12 months children (EFSA, 2009). Furan levels of 0.23-1.77 g/kg bw/day have also been found in newborns (Minorczyk et al., 2011). In adults, coffee is the most common source of furan (EFSA, 2009). In babies, the most common source of toxicants furan is either nursing or formula feeding (Lehmann et al., 2018). Wegener and López-Sánchez (2010) found high quantities of furan in prune juices, baby carrot juices, and nutritional drink flavors. It has been reported that high quantities of furan found that rye and wholegrain products. According to previous research, bottled and canned drinks are a significant source of furan for newborns (Wegener & López-sánchez, 2010). Non dietary exposure of furan can occur through inhalation during cooking. During the cooking process, (Crews, 2009) to measure the amount of furan in the air, when the cooking time was less than 10 minutes, low and fluctuating concentrations were discovered, whereas larger levels were detected when the cooking time was more than ten minutes. The amount of furan inhaled during cooking was calculated by the authors and found to be

variable depending on the food, processing time, and kind of processing, between 11 and 523 ng furan method of processing (Knutsen *et al*., 2017). However, the authors cautioned that these figures should only be used as a guide. Numerous cases of furan poisoning have been reported as a result of cigarette smoking (Pouli *et al*., 2003).

Furan induced toxicity in organism

Furan is considered harmful for its act of being carcinogenic to rats and mice. However, furan has some effects on humans as well. Furan is considered as "probably carcinogenic to humans" in a research reported by IARC (Calaf *et al*., 2020; Uçar & Pandir, 2017). It was founded by European Food Safety Authority that furan effects negatively on animals as well as humans is responsible for initiating various types of cancer (ESFA, 2017). Furan effects easily so that it has a capability to pass through membranes because of its low polarity (Abd El-Hakim *et al*., 2018).

Human have been exposed on large scale while preparing food, it might enter the human body by ingestion and inhalation. Furan is a human-toxic and carcinogenic chemical that poses a serious risk to babies (Javed *et al*., 2021). Many reports have showed that furan cause adverse effect on the vital organ of rodents (S. Gill *et al*., 2011). Furan metabolites are another source of toxicity (Bakhiya & Appel, 2010b). Furan causes liver necrosis and DNA damage in mice, which has been associated to rise up blood alanine aminotransferase (S. Gill *et al*., 2011; Mcdaniel *et al*., 2012). In rats, furan exposure raises malondialdehyde (MDA) levels while lowering glutathione-S- and superoxide dismutase, transferase (GST), catalase, (Humaira Rehman *et al*., 2019a).

The literature does not have enough information about harmful mechanism of furan on the male reproductive system. Furan altered the histological structure of ovarian cells, their DNA structure, antioxidant enzyme activity and malondialdehyde levels, (Uçar & Pandir, 2017). Furan has been shown to have antiandrogenic properties in some research (Cooke *et al*., 2014; Karacaoğlu & Selmanoğlu, 2010). Furan exposure resulted in reactive oxygen species, which damaged lymphocytes and sperm cells in human research

(Pandir, 2015a). According to a clinical investigation, higher furan and dioxin level in breast milk leads to increased thyroid hormone level in newborn babies up to 11-week-old babies (Pluim *et al*., 1993).

Furan exposure by breast feeding or food causes problems in the fetus because such chemical substances can easily pass-through placental barriers, according to previous research (Van Wijnen *et al*., 1990). The number of Leydig and germ cells, testosterone levels and sperm count decreases consequently when adult rats are exposed to furan. Moreover, it is also responsible for induction of apoptosis and spermatogenesis was hindered (Cooke *et al*., 2014; Karacaoğlu & Selmanoğlu, 2010).

Furan and Induction of Oxidative Stress

Rise in reactive oxygen species (ROS) more than antioxidant capability of cell leads to oxidative stress (Kaya *et al*., 2019). According to a report on physiology, oxidative stress (OS) leads to infertility in males (Moreiraa *et al.*, 2022). Endogenous reactive oxygen species (ROS) are formed in a different biological and chemical interactions (Azzi *et al*., 2004). When the antioxidant level is low, however, excessive ROS generation is harmful (Embola *et al*., 2002). The generation of ROS rise lipid peroxidase, which alters the acrosome reaction, fusogenic capacity and sperm DNA damage (Ichikawa *et al*., 1999). Excessive ROS damages gonadotropin receptors, lowers cyclic AMP, and decreases steroid release through damaging corpora lutes (Aten *et a*l., 1992). Result of oxidative stress approaches to spermatozoa as well because unsaturated fatty acids are present in membranes (Robert J. Aitken *et al*., 2015). ROS has caused tissue damage by interacting with biological macromolecules and producing oxidized chemicals (Elsayed *et al*., 2021). Mitochondria are found in spermatozoa and provide an endless supply of energy (Robert J. Aitken *et al*., 2015). Inner and outer mitochondrial membranes are destroyed when (ROS) are generated in excess which leads to trigger apoptosis due to release of cytochrome C consequently (Thuillier *et al*., 2003). About 15% of couples of reproductive ages have infertility. In approximately half of all cases of infertility, the male gender is too responsible. In vivo and in vitro, defective human sperm activity has

been linked to indications of increase levels of (ROS) and a loss of fertilizing capacity (Cassina *et al*., 2015). As a result, spermatozoa lack intrinsic antioxidative protection provided by ROS scavengers such as glutathione peroxidase superoxide dismutase and catalase. Non-enzymatic molecules such as vitamins C and E or glutathione are also affected. Vit C and E are highly reactive to harmful effects of oxidants in all aerobic cells (Henkel, 2011).

Since then, according to numerous studies ROS are linked to sperm dysfunction, including decreased motility, aberrant morphology, and sperm–egg penetration (Cassina *et al*., 2015). Antioxidant enzymes safeguard living cells by reducing ROS generation and detoxifying ROS through the conversion of hydrogen peroxidase (HQ) and dismutation. Through the process of dismutation, the superoxidase dismutase (SOD) creates H_2O, whereas Catalase (CAT) transforms H_2O_2 to water (Wiseman & Halliwell, 1996). In the living body, polluted substances usually target the liver since chemical breakdown occurs there, resulting in damaged hepatocytes and liver toxicity (Patel *et al*., 2012). Furan has a low polarity which allows it to be easily absorbed by the body because it is capable of passing through membranes (Bakhiya & Appel, 2010b; Crews & Castle, 2007). Exposure to this molecule is increasing every day because of its creation in home overdone food and infant goods, according to reports. Human morning urine samples have also been found to contain furan in various amounts (Ghosh *et al*., 2015). According to previous findings, the liver and kidney are the primary targets of furan toxicity. Furan was discovered to be harmful to the hepatocyte, pancreatic, and renal cells. CAT converts ROS to H_2O, and then converts H_2O to water, shielding cells from ROS toxicity (Syed & Mukhtar, 2012). If epigenetic modifications are directly linked to the action of furan, furan has a harmful effect on the pancreas by causing oxidative stress (El-Habiby *et al*., 2017).

Reproductive Toxicity Induced by Furan

Reproductive toxin can be a chemical or physical agent that can be in our environment or workplace (Babazadeh *et al.*, 2010). Many contaminants, pesticides, and dietary additives

are all known to be harmful to the reproductive system (Karacaoĝlu & Selmanoĝlu, 2010). Besides pesticides and dietary additives, furan may also affect reproductive system in male rats. Furan induces toxicity in endocrine and reproductive system in male rats (H. Rehman *et al*., 2020). Exposure of male rats to furan results as decreased testosterone level. Furan also affects male rats as decreasing luteinizing hormone level significantly. High dose of furan (up to 8 milligram per kilogram body weight) is responsible for a significant decrease in weight of seminal vesicle and a significant increase in weight of prostate gland. However, there is always a factor of inconsistencies which may alter the impact of furan on prostate, testes, and epididymis. Furan changes the lumen diameters and epithelial heights after rats are exposed to furan. Moreover, it is also responsible for an increase in apoptotic cells of testis (Karacaoĝlu & Selmanoĝlu, 2010).

According to these investigations, individuals are exposed to furan in a variety of ways, both directly and indirectly. As a result, an appropriate approach must be used to conduct a furan risk assessment on mammalian reproductive toxicity. Furthermore, there is minimal information on the potential negative effects of furan on the reproductive system (EFSA, 2009). However, due to the reproductive system's complicated structure, regulatory activities, and physiology, there is a considerable potential that toxicants will cause a variety of serious toxicological problems at multiple places throughout the reproductive tissues and organs (Creasy & Foster, 2002).

Recently, in an epidemiological investigation, breast milk and placenta of human was reported to be contaminated with furan (Li *et al*., 2009; Shen *et al*., 2015). Infertile males' semen allegedly contained higher quantities of furans than fertile ones implying a link between furan exposure and reproductive function (Galimova *et al*., 2015). Furan has been demonstrated to produce ROS, linked to human lymphocytes, DNA damage and sperm cells (Pandir, 2015a). As a result, excessive human exposure to furan in the diet and environment is a public and scientific problem (Sirot *et al*., 2019).

It is enclosed in previous studies that animals more frequently exposed to furan are more like to receive a negative impact on their reproductive systems. Consequently, affected animals have to bear a decreased in daily production of sperm count. In addition, their estrous cycle also gets disrupted (Humaira Rehman *et al*., 2020). Rats exposed to 10 to 20 milligram per kilogram furan during the stages of their reproductive development received a negative impact on reproductive development (Humaira Rehman *et al*., 2019b). Furan seriously affects Leydig cells and spermatogenesis as it is responsible in induction of apoptosis in Leydig and germ cells because furan is antiandrogenic (Karacaoĝlu & Selmanoĝlu, 2010).

Currently, there is a lack of information and study on the real mechanism of furan's toxicological effects on the reproductive systems. However, based on prior observations and studies on heat-induced food toxicants, we may infer that furan will also impair reproductive performance in terms of reproductive physiology and endocrinology dysfunction, such as the HPA-Axis, HPG-Axis, and HPG-Axis.

There are not as many studies on chemoprotection of furan induced toxicity in male reproductive system yet, but a few of them demonstrated that negative impacts of furan on male reproductive system such as slowing down catalytic activity is alleviated by the use of garlic oil. Garlic oils also regulates the down regulated cytochrome-P450 and caspase-3 and damaged testicular oxidation level in hormone (El-Ekabawy & El-Sherif, 2016). In addition, *Spirulina platensis* was found to reduce furan-induced reproductive damage in rats' testes by reducing oxidative stress and inflammation. *Spirulina platensis* is known as blue green algae. It is edible and used to cure apoptosis (Abd El-Hakim *et al*., 2018).

Palliation Factors of Furan

Food industries have been implicated of employing various mitigation measures that may have an impact product safety and nutrition effects. Knowingly, each used method has its own consequences, such as it should not exceed the limit to which it is acceptable and what sensory attributes are desirable (Kettlitz *et al*., 2019). There are various techniques

used for significantly reducing such as modifying precursor level, change in conditions of thermal processing, temperature approach and high-pressure techniques. For most significant reduction antioxidants are added to food system (Batool *et al*., 2021).

The most effective way of significant furan reduction is adding gallic acid and malic acid in food juices along with UV-treatment. Gallic acid and malic acid act as antioxidants. Antioxidants are effective in furan mitigation as it stops the reactions responsible for furan formation such as free radical chain reaction (Hu *et al*., 2018).

As a result of delayed lipid oxidation, various investigations have shown that some antioxidants are promising variables for furan reduction. Various polyphenolics (tyrosol, punicalagin, chlorogenic acid, ellagic acid, epicatechin, caffeic acid, and oleuropein) are also used as antioxidants to inhibit induction of furan (Bi *et al*., 2017). According to current study Vit C is used as protective measure to mitigate the toxicity induced by furan in male Sprague Dawley rats.

Antioxidant

Antioxidant mechanisms are present in the human body to remove free radicals (Hamidian *et al*., 2020). Cells rely on numerous extrinsic and intrinsic antioxidant systems which are capable of combating with elevated levels of ROS The non-enzymatic antioxidant such as glutathione as well as the enzymatic antioxidant like thiol peroxidase, glutathione and catalase are the example of endogenous antioxidant. Exogenous antioxidants, on the other hand, are micronutrients including vitamin A, Vit C, Vit E, N acetyl cysteine, L carnitine and trace elements such as selenium and zinc that must be provided to the body exogenously in order to keep a fine balance between oxidation and reduction (antioxidation) in any living cell (Gutteridge & Halliwell, 1989; Valko *et al*., 2007). An imbalance between production of free radical and capacity of antioxidant is caused oxidative stresses (OS). which impairs the structure, function, and motility of spermatozoa, resulting in male infertility (Rafiee *et al*., 2016). Dietary supplementation with antioxidants improves sperm cell motility and adequate sperm concentrations (Sheweita *et al*., 2005).

Vitamin

Vitamins, as key antioxidants, are important for removing free radicals from the body (Raeeszadeh *et al*., 2022). Vitamins are required for regular metabolic activities and equilibrium in the human body. Vit C and Vit E are antioxidants with a low molecular mass that removed free radicals (Janisch *et al*., 2005). Natural antioxidant is very useful in protecting the negative effect of in the quality of sperm in testes (Yousef, 2010). The Institute of Medicine (OM) recognized Vit C and E as dietary antioxidants in 2000 and evaluated data that these nutrients can help prevent or treat several chronic diseases. A dietary antioxidant is a food component that reduces the negative consequences of reactive species in humans, such as nitrogen species and reactive oxygen, on normal physiological functioning (Lobo *et al*., 2010).

Vitamin C

Ascorbic acid (AA) is considered as most effective antioxidant. Ascorbic acid is a form of Vit C found in biological system (Zhitkovich, 2021). In 1923, a Nobel laureate known as Szent-Gyorgyi was a Hungarian biochemist who isolated ascorbic acid as Vit C. However, ascorbic acid was synthesized by Howarth and Hirst (Millar, 1992). Furthermore, it is required for the manufacture of collagen, L-carnitine, and norepinephrine. Humans cannot produce Vit C thus they must obtain it through their diet. As a result, long-term Vit C deficiency causes problems with collagen post-translational modification, which leads to sickness and death. Vit C deficiency has been linked to several diseases that involve increased oxidative stress, including diabetes, cancer, and cataracts. AA is a non-fat soluble antioxidant that can help to decrease the effects of free radicals (Ahmed Talat Galal, 2022). Even though AA is easily soluble in water, it can recover both outside and inside damage of cell by free radical (Costa *et al*., 2016). AA is preventing the toxic effect of oxidant by reducing the reactive ROS and nitrogen species (NS) into stable compound and act as cofactor in the enzymatic biosynthesis, catecholamine, carnitine, peptide neurohormones and collagen (Wilson, 2002). Moreover, it is required for the manufacture of collagen, L-carnitine, and norepinephrine.

Humans cannot produce Vit C, so they must obtain it through their diet. Consequently, long-term Vit C deficiency causes problems with collagen post-translational modification, which leads to sickness and death. Vit C deficiency has been linked to several diseases that involve elevated oxidative stress, such as diabetes, cancer, and cataracts. Infection is occurring due to HIV as well as smoking habits (Duarte & Lunec, 2005). Neutrophils are phagocytic cells. Vit C is aggregated in neutrophils. Vit C is helpful to improve phagocytosis, chemotaxis, ROS formation and microbial death. Vit C is also considered as an effective remedy to cure macrophage clearance in sites where neutrophils are wasted and most importantly for apoptosis. Vit C is also crucial for necrosis reduction. It also cures potential tissue injuries (Carr & Maggini, 2017).

Spermatogenesis has been protected by Vit C, which play critical role in the fertility and semen integrity in both men and animals (Eskenazi *et al*., 2005) as well as enhance testosterone levels and prevent sperm agglutination (Sönmez *et al*., 2005). It's a major chain breaking antioxidant, accounting for up to 65 percent of the total antioxidant capacity of intracellular and extracellular seminal plasma (Makker *et al*., 2009). Use of a diabetic mouse model, (Shrilatha & Muralidhara, 2007) studied the protective benefits of Vit C on testicular stress, sperm oxidative stress, and genotoxic impacts. Similarly, the Naziroglu (Naziroğlu, 2003) have been reported that Vit C act as important antioxidant against oxidative stress.

Aims and Objectives

Furan has toxic effect on male rates reproductive system, the current study was designed to emulate the effect of furan on male rat with the help of Vit C. Aims and objectives of the study are:

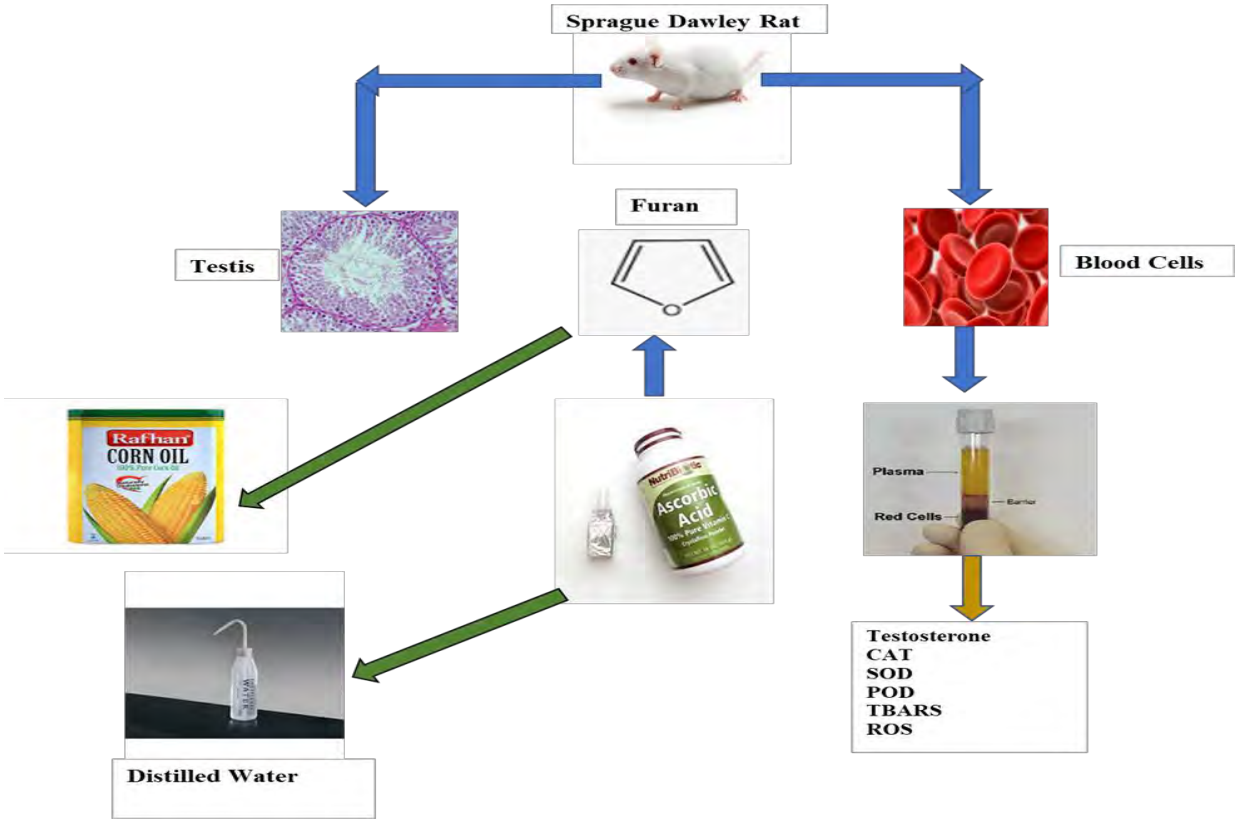
To monitor the histomorphological changes induced by furan and Vit C in testes

- To evaluate the protective effects of Vit C in adult male rats after exposure to furan by measuring oxidative stress and histological examination
- To assess the antioxidant potential of Vit C against furan treated rats
- To find out the production of ROS and acidic species in furan and Vit C treated rats
- To understand the functional capacity of Leydig cells in furan and Vit C treated adult Sprague Dawley rats by measuring plasma testosterone levels.

Materials and Method

Animals and chemicals

In the current study we were used 20 Sprague Dawley adult male rats, Animals were handled in accordance with guidelines and rules approved by the Department of Animal Sciences' local ethical council, which are research-specific related to animal handling. The rats were kept in special steal cages and were fed both water and ad libitum with 12-hrs dark and light cycle at 25ºC. The research study was permitted by the Institutional Ethics Committee of Quaid-I- Azam University Islamabad Pakistan.



Chemicals

Corn oil was used to prepare furan (CAS 110-00-9, Sigma- Aldrish Poole, Sigma Aldrish Co Ltd, Dorset, UK) on the time of use and stored brown bottles with proper sealing. Furan was orally administered through gavage in corn oil of 30 mg/kg concentration. Ascorbic acid CAS-No: 50-81-7, Sigma-Aldrich Poole, Sigma Aldrich Co. Ltd, Dorset, UK) was used. Doses were made individually in volume-to-weight ratio (v: w) and stored in the refrigerator after proper sealing with plastic cessations and transformed silicon septa) till fourteen days (according to earlier findings related to stability of furan (NTP, 1993). The same protocol was followed again after 14 days. Furan was dissolved in corn oil vitamin C was dissolved in distilled water before being given to animals.

Study designation

The rats were randomly categories into total four groups, each with 5 rats kept in steel cages with weight range of a rat from 150 and 200 gm. A sub-chronic investigation was carried out for total 28 days to check the therapeutic effect of Vit C against furan-induced damage to male rat's reproductive systems.

The doses were orally administered at a precise time between 11am and 12 am. The experimental rats were randomly divided into four main categories:

Group I: Rates that received corn oil only.

Group II: The animals were received 30 milligram/kilogram of dissolved furan in corn oil.

In Group III: The rats were received 200 mg/kg of vitamin C dissolved in distilled water.

In Group IV: The animals were administered with 30 mg/kg of furan and 200 mg Vitamin C.

All the animals were sacrificed at the end of experiments testes were removed and blood was taken through decapitation, and both were used for further biochemical studies. The level of antioxidant enzymes like SOD, CAT, POD, ROS, TBARS and testosterone

levels were also studied in the plasma of the blood. Histopathologic examination was performed by a light microscope.

Tissue collection and Blood

The experiment was carried out for 28 days. Animals were weighed and decapitated on day 29. Heparinized syringes were used to collect trunk blood directly after decapitation and preserved in heparinized tubes. The samples were then centrifuged at 3000 rpm for 15 minutes. The blood plasma was collected and stored at -20°C for additional studies. The entire animal's testicular and epididymal tissues were collected. For histological analyses, in 10% formalin right epididymis and testicular tissues were fixed under microscope.

Biochemical Analysis

For biochemical parameters and hormonal levels, Plasma was collected and stored at -20ºC. The frozen samples were thawed and were used for antioxidant status and hormonal levels of control and treated mice were measured.

Superoxide Dismutase (SOD)

SOD activity was checked using the protocol of (Kakkar et al., 1984)

Procedure:

0.1 ml of phenazine methosulphate (186 μM), 1.2 ml of sodium pyrophosphate buffer (0.052 mM at pH 7.0) and 0.3 ml of sample, were mixed and 0.2 ml of NADH (780 μM) was added to start the reaction. Finally, after one minute the reaction was ended with the adding of 1 ml of glacial acetic acid. The readings were noted at 560 nm and the results were measured as protein in units/mg.

Catalase (CAT) activity

The method was utilized to determine the activities of CAT with small modifications (Chance & Meahly, 1955)

Reagents:

PBS 25 ml (50 mM pH 5) .H_2 O, 0.4 ml (5.9 mm)

Procedure:

The 0.1 ml of blood plasma, 0.4ml of 5.9 mM hydrogen peroxide and 2.5ml of 50 mM phosphate buffer pH; 5.0 were taken in a cuvette to determine the CAT levels in blood plasma. After one minute, the difference in absorbance of solution at 240 nm was noted. In 1 minute, the 0.01 units change in absorbance was considered as 1 unit CAT activity.

Estimation of guaiacol peroxidase activity (POD)

By following Chance and Machly (1955) protocol, with small modification was utilized to determined POD levels

Reagents:

0.3 milliliter of 40 Mm H_2O_2

0.1 milliliter of 20 mM guaiacol

2.5 milliliter of 50 mM PBS (pH: 5)

Procedure:

Reaction was done by adding of 0.1 ml of 20 mM guaiacol, 2.5 ml of 50 mM PBS (pH:5.0), and 0.3 ml of 40 mM H_2O_2 into 0.1 ml of homogenate**.** Absorbance was observed at 470 nm after 1 minute. The change in absorbance of 0.01 units in 1 minute was termed as unit of POD activity.

Analysis of Reactive oxygen Species Assay (ROS)

By following Hayashi *et* al (2007) protocol, reactive oxygen species was determined in plasma of control and treated group.

Reagent:

0.06 ml homogenate

0.05 ml of 0.1M ferrous sulphate

0.1 M; sodium acetate buffer (pH: 4.8)

10 mg DEPPD (N, N- Diethyl-p-phenylenediamine sulphate salt)

Procedure:

500 milliliter of distilled water and 4.1 gm of sodium acetate (CH_3COONa) was dissolved to make 0.1 M sodium acetate buffer (pH 4.8). 10 mg N, N- Diethyl-p-phenylenediamine sulphate salt was melted in 100 ml CH_3COONa. And the second solution was made by adding of 50 mg of ferrous sulphate in 10 milliliter of CH_3COONa. The above prepared solution was mixed at a ratio 0f 1: 25 incubated for 20 minutes in dark condition at a room temperature. Then 20 µl of solution mixture, 20 µl homogenate and 1.2ml buffer were taken in a cuvette and absorbance was measured at 505 nm through TM plus spectrophotometry. After every fifteen minutes, three reading were taken for each sample.

Thiobarbituric acid reactive substances (TBARS)

The method was used to estimate lipid peroxidation with some modification (Colby *et al*., 1981)

Reagents:

PBS	0.58 ml (0.1 M, pH: 7.4)
Ascorbic Acid	0.02 ml
Ferrie Chloride	0.02 ml
Trichloroacetic	1 ml (10%)
Thio-barbituric acid	1 ml (0.67%)
Plasma	0.2 ml

Procedure:

The reaction solution was composed of 0.2 ml of sample, 0.2 ml vitamin C of 100 mM, 0.02 mM FeCl of 100 mM and 0.58 ml phosphate buffer of 0.1 M (pH;7.4) at 37 °C in a water bath. The prepared mixture was allowed to incubate for one hour and 1 ml of trichloroacetic acid (10%) was utilized for completion of the reaction. Then, 1ml of

0.67% TBARS was added, and all tubes were held in a water bath of boiling water for only 20 minutes before positioned into a crumpled ice-bath. After that, the sample was allowed to centrifuge at 25000 rpm for 15 min, and readings at 535 nm were measured by using spectrophotometer.

Tissue Histology

Histology of testicular and epididymal tissues was used to investigate the antifertility impact of furan on the testis and epididymis. Following the collection of testicular and epididymal tissues, the following stages were carried out.

Placed tissue in Sera for 4-6 hours

Placed tissue in 10% formalin for 24-48 hours

Dehydration

Tissues were dried at room temperature in ascending grades of alcohol after fixation as given below.

In 70% Ethanol for 1 hr

For one hour in 80% Ethanol

For one hour in 90% Ethanol

For one hour in 95% Ethanol

For one hour and 30 minutes in 100% Ethanol

For one hour and 30 minutes in 100% Ethanol II

For one hour and 30 minutes in 100% Ethanol III

Embedding

Following steps were taken for embedding. Tissues were kept in xylene and fixed in paraffin in following order.

For one hour In Xylene I

For one hour In Xylene II

In Paraffin I (58°C) for two hours

Dehydration

Tissues were then melted and placed on a boat. The wax was allowed to become hard after removing all bubbles. Tissue-filled paraffin wax blocks was cropped and fixed on blocks of wooden before being sliced into sections with a knife or scalpel.

Preparation of Albumen Slide

1. Two egg whites were added to 1200mL of deionized water.

2. Followed by stirring with the help of a magnetic stirrer for just 5 minutes.

3. Then, 4 ml of concentrated NH_4 OH was added.

4. Again stirred for 5 minutes.

5. Followed by filtering with the help of low-grade filter such as coffee filter.

6. Stored albumin in a "screw-top glass" bottle under dark condition at 40˚C.

Coating Slide

1. For coating slides, slides were individually laid out onto a slide heater at a lower setting.

2. By using a small new clean paintbrush each slide was painted by a thin layer of albumin. The process is repeated at least three times.

3. Slides were allowed to dry onto the heating plate at a lower setting for overnight.

4. Albumin-coated slides were kept in the original packaging at room temperature until needed.

Microtomy

After embedding, the paraffin-embedded tissues with molten wax were placed on wooden blocks. The tissue was cut into seven cm thin pieces using a microtome (Thermo, Shandon finesse 325, UK). And then the lengthy ribbons tissues were extended before being put in previously prepared clean albumenized glass slides. These slides were kept

heated at 60°C on a Fischer slide heater. Glass slides were then positioned in an incubator for a night of stretching**.**

Staining

Slides were stained in different grades as follows:

1. For 3 minutes in Xylene I
2. For 3 minutes in Xylene II
3. For 3 minutes in 100 % alcohol I
4. For 3 minutes in 100 % alcohol II
5. In 90% alcohol for 3 min
6. In 70% alcohol for 3 min
7. Washed with water for 3 min
8. Hematoxylin for 8 min
9. Washed with water for 2 minutes
10. In acidified alcohol for 1 minutes
11. Washed with water for 2 minutes
12. In bluing solution for 2 minutes
13. Washed with water for 2 minutes
14. In 90% alcohol for 10 dips
15. In Eosin for 2 minutes
16. Washed with water for 2 minutes
17. In 90 % alcohol for 5 minutes
18. For 5 minutes in 100 % alcohol I
19. For 5 minutes in 100 % alcohol II

20. For 5 minutes In 100 % alcohol III

21. In Xylene I for 5 minutes

22. In Xylene II for 5 minutes

23. To per-mount, DPX with three small drops were added and cover slip was placed before drying of paramount.

Light Microscopic Study

Five cm thick tissue sections were examined at 40X magnifications using a light microscope (Nikon, 187842, Japan). All the experimental groups slides were examined. The following parameters were evaluated for histomorphometry analysis utilizing the picture J2x package software programmed luminal diameters and seminiferus tubular, epithelium height of testicular tissue and Tubule diameter, lumen, and epididymal tissue epithelial height were also measured by using image J2x

Microphotography:

The microscopy was done by utilizing Leica LB (Germany) microscope armed with a digital cannon camera (Japan).

Hormonal Analysis

Quantitative Analysis of Testosterone Concentration

Testosterone concentrations were found out quantitatively by utilizing enzyme immune-assay (EIA) kits (Bio check Inc, USA). The principle of the assay is as follows.

Hormonal Analysis

The Quantitative Determination of Testosterone Concentration kit (PerkinElmer Inc, USA) was used to determine the Testosterone level quantitatively.

The principle of the assay as follow

Principle of the Assay

In the Enzyme Immuno Assay, the testosterone enzyme is employed to build a competitive link between a fixed amount of rabbit anti-testosterone in test samples and the Testosterone HRP-conjugate. At 30 degrees Celsius, the goat anti-rabbit IgG- The coated wells with goat anti-rabbit IgG were incubated at 30ºC for 60 minutes along with 100 ul HRP conjugate reagent of testosterone, 50 litter rabbit anti-testosterone, 25 ul testosterone, patient samples and standards.

During incubation, a given amount of testosterone labelled with HRP competes for the number of permanent binding sites on a specific testosterone antibody with the quality control serum or standard sample endogenous testosterone. A measured amount of HRP-labeled testosterone contends with the reference sample or standard sample endogenous testosterone during the incubation for the same number of binding sites as the testosterone's specific antibody. Then removed unbound testosterone peroxidase conjugates and TMB Reagent solution was poured and incubated for 20 min at room temp until the solution becomes blue.

The mixing of 2N HCL terminates the color development and determines the absorption at 450 nm spectrophotometrically. The amount of enzyme present in the sample dictates the color intensity produced, which is in reverse proportional to the unlabeled amount of testosterone. By comparing the standard concentration to the absorption, a standard curve is produced. The standard curve can be used to determine the testosterone concentration in specimens and controls that are operated at the same time by the standard.

Procedure:

First, 25µ*l* was distributed into the appropriate wells of samples, controls, and standards after grading the holder with the necessary number of coated wells. After that, each well received 50µl rabbit anti-testosterone. It was important thoroughly well mixed for 30 seconds. The reagent was filled with 100µl testosterone-HRP conjugate reagent after 60 minutes at 37°C. The substrate was washed and then flashed the specified number of micro-wells using a washing buffer (1x) 100ul. TMB was then removed and gently mixed

for 10 seconds, followed by a 20-minute incubation period at room temperature. We added 100µl of stock solution into each well for stopping the reaction and then stirred it gently for 30 seconds. This step is very important to convert full blue color into yellow color. Finally, using a 15-minute microtiter well reader, absorbance was noted at 450 nm.

Statistical Analysis

Graph pad Prism software was used to perform an ANOVA ("one-way analysis of variance"). It is a statistical technique for calculating the (ANOVA)) tracked by a Dunner's test that is used to compare different groups. All calculated values were expressed as Mean SEM. "The degree of significance was fixed to $p < 0.05$".

Results

Body weight

Significant alteration in body weight was seen in all groups as shown in table 1.

Multiple t-test of body weight of the rats was conducted to examine the body weight change of rats. There was no significant change in body weight of rats test result although a small increase in the weight of each group was seen.

Table 1: Shows the 28-day Mean ± SEM body weight (g) of adult male rats in the control and treated groups

Group 1	Control	Furan	Vit C	Vit C + F	p-value
Day 01	188.60±7.58	187.40±8.74	169.40±9.90	191.30±8.47	p=0.0208
Day 28 Treatment	217.30±8.93	211.30±7.58	186.60±8.03	213.10±9.713	p=0.0007

All values are shown as Mean $\pm$ SEM

* P<0.05 for control group only.

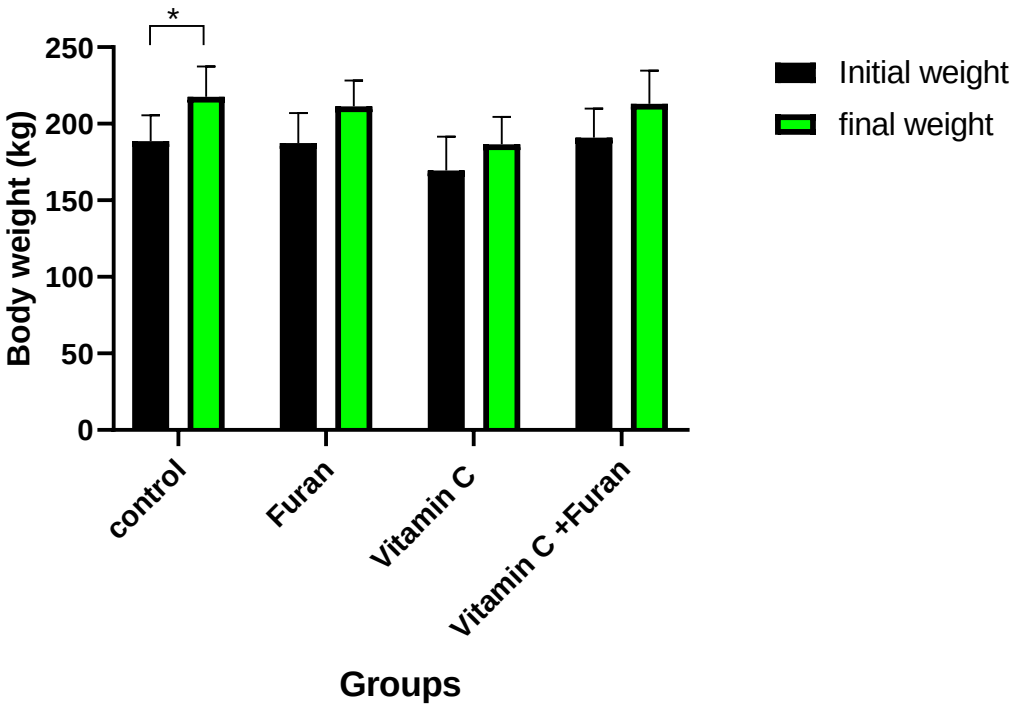


Figure 3: Body weight, in terms of initial and final value, was compared for each group by using multiple t-tests. Final body weight was documented significant increase ($p<0.05$) as compared to initial body for control group. No significant difference was seen in other groups.

Organ weight

One way ANOVA test was conducted to determine the testicular weight change of the rats as shown in Table 2. The results clearly showed that there was no significant change in testicular weight. On examining the epididymal weight, it was concluded that there was a significant change in epididymal weight of each group (i.e., $p<0.0469$).

Table 2: Mean ± SEM testicular weight (g) and epididymis weight (g) of treated and control adult male rats after 28 days of treatment.

Parameters	Control	Furan	Vit C	Vit C+ F	p-value
Testicular weight (Right + Left)	2.5 ± 0.13	2.30±0.19	2.19±0.27	2.59 ± 0.084	P=0.4426
Epididymal weight (Right + Left)	1.74±0.10	1.28 ± 0.16	1.21±0.06	1.22 ± 0.05	P=0.0421

The values are stated as Mean $\pm$ SEM. *, **, ***, **** showing significant variance at $p < 0.05, p < 0.01, p < 0.001$ and $p < 0.0001$ respectively

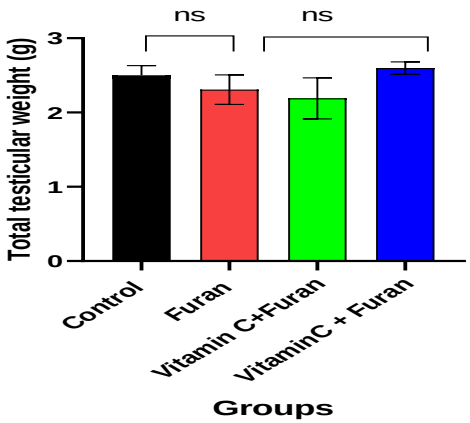


Figure 4: Combined testicular weight was compared between all the four experimental groups by using one-way ANOVA followed by Tuckey's test. No significant difference was observed among all the four groups.

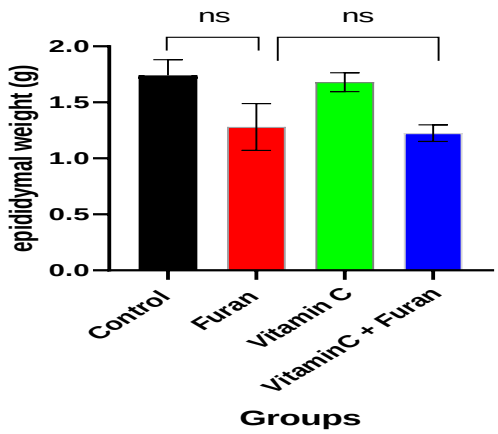


Figure 5: Combined epididymal weight was compared between all the four experimental groups by using one-way ANOVA followed by Tuckey's test. Significant difference was observed in control and Vit C as ($p < 0.05$). No significant difference was observed among all the four groups.

Biochemical Parameters

Antioxidant

Mean ±SEM specific activity in the control and treated group for antioxidants, including peroxidase (POD), catalase (CAT), superoxidase (SOD) thiobarbituric acid (TBARS) and reactive oxygen species (ROS) has shown in Table 3.

Catalase (CAT)

The CAT activity in plasma of group treated with furans and control. As opposed to the control (****$p<0.0004$) and Vit C-treated groups, CAT levels were considerably lower in the furan-treated group (**$P< 0.01$).

Decreased levels of CAT were recovered significantly (**$p<0.01$) in Vit C+F group as compared to furan treated animals.

Superoxide Dismutase (SOD)

In compared to the control group the SOD level considerably decreases (***p0.0003) in furan-treated groups. The increased level of SOD was significantly recovered after treatment with Vit C.

Peroxide (POD)

POD levels were also affected by the dose of furan as a significant decrease in POD level was seen after treatment with furan as compared to control group. Vit C played a vital role in recovering the decreased levels of POD significantly (*p<0.0256).

Reactive Oxygen Species (ROS)

ROS levels due to a dose of furan increased significantly (***0.0001) as compared to control group. ROS levels were significantly recovered after treatment with Vit C.

(TBARS)

Level of TBARS was also affected by furan treatment. Furan caused an increase in TBARS level. The increase caused by furan was significant (***p<0.0003) and was recovered significantly with a dose of Vit C.

Table 3: Means ± SEM biochemical parameter in control and treated male rats after 28 days of treatment, respectively.

Parameters	Control	Furan	Vit C	Vit C + F	P-Value
CAT (U/min^{-1} Protein)	7.90 ± 0.02	4.34 ± 0.19^{a***}	6.78 ± 0.49^{b**}	5.04 ± 0.09	p<0.0004
SOD (U/mg^{-1} Protein)	36.18±0.63	27.10 ± 0.98^{a***}	35.122±0.89^{b***}	33.37±1.01^{b**}	p<0.0003
POD (n mole)	9.90±0.50	6.89 ± 0.47^{a*}	9.76 ± 0.63^{b*}	8.69 ± 0.74	p<0.0256
ROS µmol/min	0.78 ± 0.01	1.02 ± 0.01^{a***}	0.75 ± 0.01^{b***}	0.80 ± 0.02^{b***}	p<0.0001
TBARS nmol/mg	17.19 ±0.09	23.42±0.49^{a***}	17.34±0.66^{b***}	16.72 ± 0.55^{b***}	p<0.0003

The values are stated as Mean $\pm$ SEM. *, **, ***, **** showing significant variance at $p < 0.05, p < 0.01, p < 0.001$ and $p < 0.0001$ respectively.

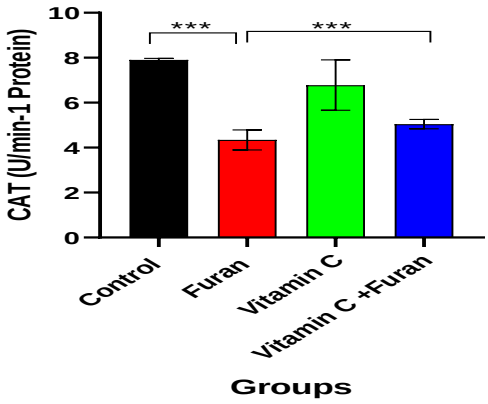


Figure 6: CAT (U/min^{-1} Protein) protein levels were compared by using one-way ANOVA in combination with Tukey's test. The level of CAT decreased significantly in furan group as compared to control. The decreased CAT level was significantly recovered by a dose of Vit C. Moreover, the test was significant among furan and Vit C+ F group.

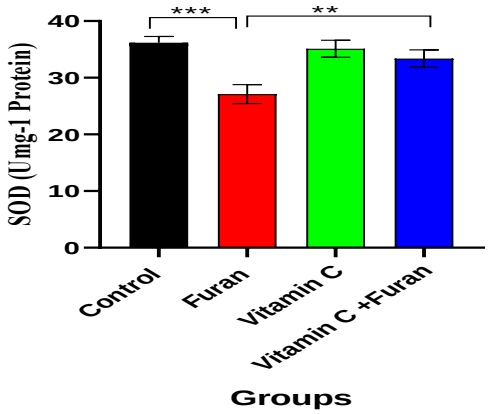


Figure 7: Comparison of SOD (Umg-1 Protein) by using one-way ANOVA followed by Tuckey's test. Superoxidase dismutase activity (U/min) was observed after 28 days of experiment. Protein levels of SOD were significantly decreased (***p<0.0001) in furan treated groups as compared to control and Vit C group. Decreased levels of SOD were significantly recovered (***p<0.0001) in Vit C+ F group as compared to furan treated group.

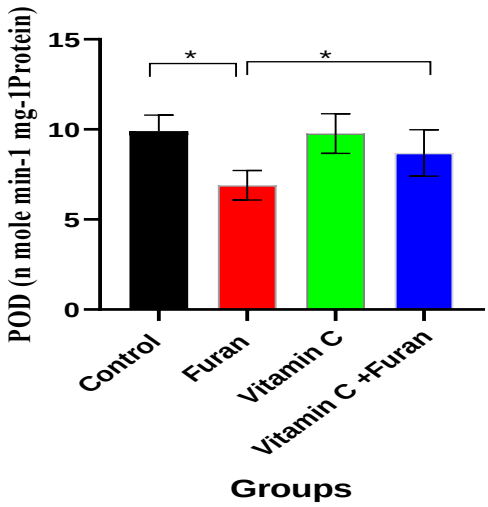


Figure 8: Comparison of POD (Umg-1 Protein) by using one-way ANOVA followed by Tuckey's test. Protein levels of POD were significantly decreased (*p<0.0308) in furan treated as compared to control and Vit C group. Decreased levels of POD were significantly recovered (*p<0.0388) in Vit C+ F group as compared to furan treated group

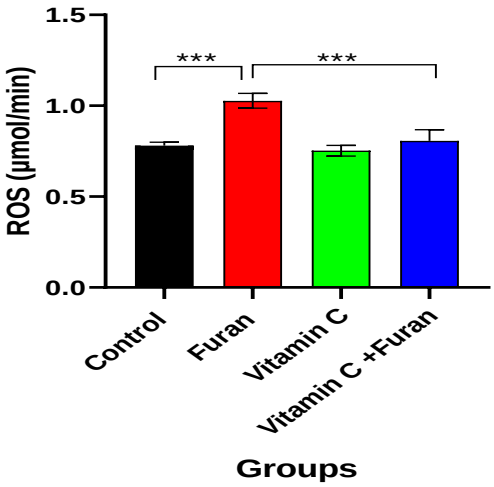


Figure 9: One-way ANOVA and Tuckey's test were used to compare ROS (mol/min) levels in the four experimental groups. Compared to the control and Vit C+F groups,

ROS levels were significantly higher in furan-treated animals (***p<0.0001). ROS levels were significantly restored (***p=0.001) in the Vit C+F groups.

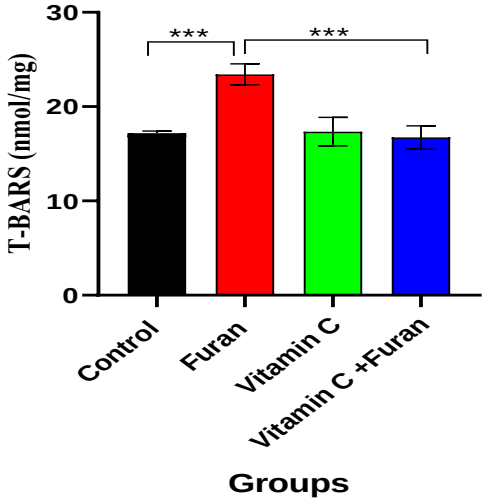


Figure 10: T-BARS (nmol/mg) levels were compared by using one-way ANOVA followed by Tuckey's test in all the four experimental groups. LPO levels were significantly increased in furan treated groups in comparison to control one (***p<0.001) and Vit C (***p<0.001). This difference was recovered significantly (***p<0.001) in Vit C+ F group animals.

Histopathological Analysis:

Testes:

Table 4 covers the histopathological parameter of the seminiferous tubule of testes in adult rats. Measurements of lumen diameter, tubular diameter, epithelial height of the testicles are included in the below table. The p-value calculated for each set of measurements shows whether the test was significant or not. If the p-value is greater than 0.05, it means that the test was not significant. If the p-value calculated for the measurements is less than 0.05, it says that the test was significant. All the p-values are measurements using one-way ANOVA followed by Tukey's post HOC test.

Tubular diameter of testicular in furan group seen significant (**P<0.0027) difference in comparison to control. The tubule diameter of testes decreased significantly when treated with furan and histopathological parameters were strongly modified causing changes in sperm count as well. The decreased tubule diameter after treatment with Vit C was recovered significantly.

Lumen diameter, after treating with furan; faced a significant (**P<0.0065) increase which was decreased significantly after treatment with Vit C.

The epithelial height of the testes was significantly altered by furan dose. In the 30mg furan group, the epithelial height of testes was considerably (****p0.0001) lower than in the control group. Vit C played a vital role in increasing the decreased epithelial height. After the dose of Vit C, the epithelial height was recovered and almost like that of the control group.

Caput Epididymis:

Table 5 covers the histopathological parameter of caput epididymis. 30mg dose of furan caused changes in measurements of tubule diameter, lumen diameter and epithelial height. The p-value calculated for each set of measurements shows whether the test was significant or not. If the p-value is more than 0.05, it means that test was not significant. If the p-value calculated for the measurements is less than 0.05, it says that the test was significant. All the p-values were measured using one-way ANOVA followed by Tukey's post HOC test.

The tubular diameter of caput in furan treated group was not decreased significantly in comparison to the control group. However, a little decrease was examined after furan treatment. Effects of furan on lumen diameter of caput were similar to the effects caused by furan on tubule diameter. The lumen diameter received a decrease after furan treatment, but the difference was too little to be significant. Same as tubular diameter and lumen diameter, furan was unable to cause a significant difference in histopathology of epithelial height of cauda. A little decreased was examined in epithelial height, but the difference was not enough to be significant.

Histopathological analysis of caput proved that furan did not effect on tubular diameter, lumen diameter and epithelial height of caput.

Cauda Epididymis:

Table 6 covers the histopathological parameter of cauda epididymis. 30mg dose of furan caused changes in measurements of tubule diameter, lumen diameter and epithelial height of cauda. All the p-values of measurements were calculated by one-way ANOVA followed by Tukey's post HOC test.

ANOVA test conducted to examine tubular diameter of cauda proved that furan was unable to change the histopathology of caudal epididymis. There was a decrease in tubular diameter of cauda after treatment of furan. However, the difference was too low to be significant. The P value calculated for tubular diameter of cauda was P=0.8441 which was not significant. Lumen diameter of cauda epididymis was increased a bit but not significantly as compared to control group after treatment with furan. However, there was a small increase in lumen diameter of cauda. The P value calculated for lumen diameter was P=0.9856 which is greater than 0.05. As it was seen that furan was unable to modify the tubular diameter and lumen diameter of cauda, but the 30mg dose of furan was enough to significantly decrease the epithelial height of cauda. The epithelium height decreased significantly (*P=0.0162) in the ANOVA test used to establish the P value, which was then improved by the dose of Vit C.

The following table covers the histopathological parameter of seminiferous tubule of testes of adult rats. Under-study groups i.e., control, furan, Vit Cand Vit C+ F can be seen in columns while measured tubule diameter of testes, lumen diameter of testes and epithelial height of testes of the groups under study can be seen in rows. The p-value calculated for each set of measurement shows whether the test was significant or not. If the p-value is greater than 0.05, it means that the test was not significant. If the p-value calculated for the measurements is less than 0.05, it says that the test was significant. One-way ANOVA and Tukey's post HOC test were used to obtain all p-values.

Table 4: Means ± SEM after 28 days, histopathological parameters of the seminiferous tubule of the testes were determined in control and treated adult male rats.

Parameters	Control	Furan	Vit C	Vit C + F	P-value
Tubular diameter (µm)	662.52±9.95	599.56±17.71^{a**}	645.15±10.87^{b*}	624.18±8.96	p<0.0027
Lumen diameters (µm)	284.97±14.36	362.29±31.72^{a*}	270.47±11.78^{b*}	273.58±17.62^{b*}	p<0.0065
Epithelial height µm	224.48±11.83	179.91±10.91^{a***}	220.30±9.36^{b***}	159.28±6.72^{a*c*}	p<0.0001

The values are stated as Mean ± SEM. *, **, ***, **** showing significant variance at $p < 0.05, p < 0.01, p < 0.001$ and $p < 0.0001$ respectively.

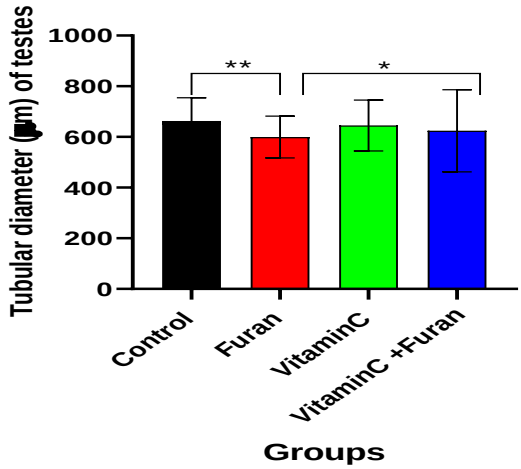


Figure 11: Tubule diameter of testes was reduced in the furan-treated group compared to control group. The test was highly significant as (**P<0.0027).

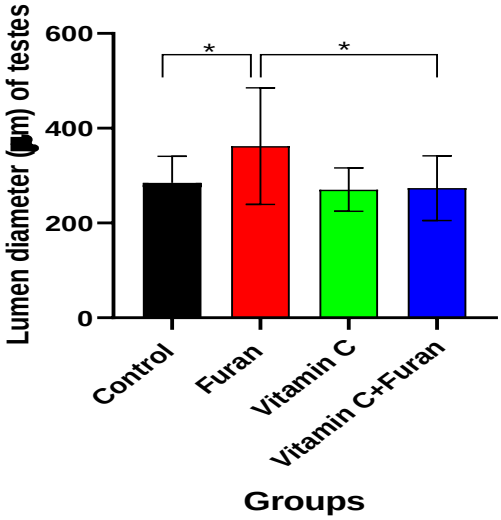


Figure 12: Lumen diameter of testes was increase in furan-treated group compared to control group. The test was significant as$(p = 0.0065)$.

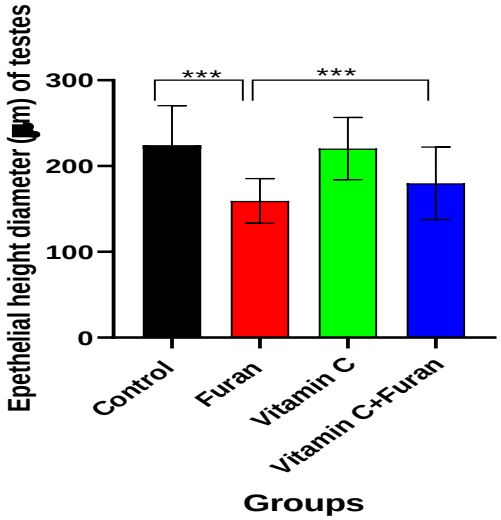


Figure 13: Epithelial height of testes was reduced in furan-treated group compared to control group. The test was highly significant as p-value was less than 0.05 (***p<0.0001).

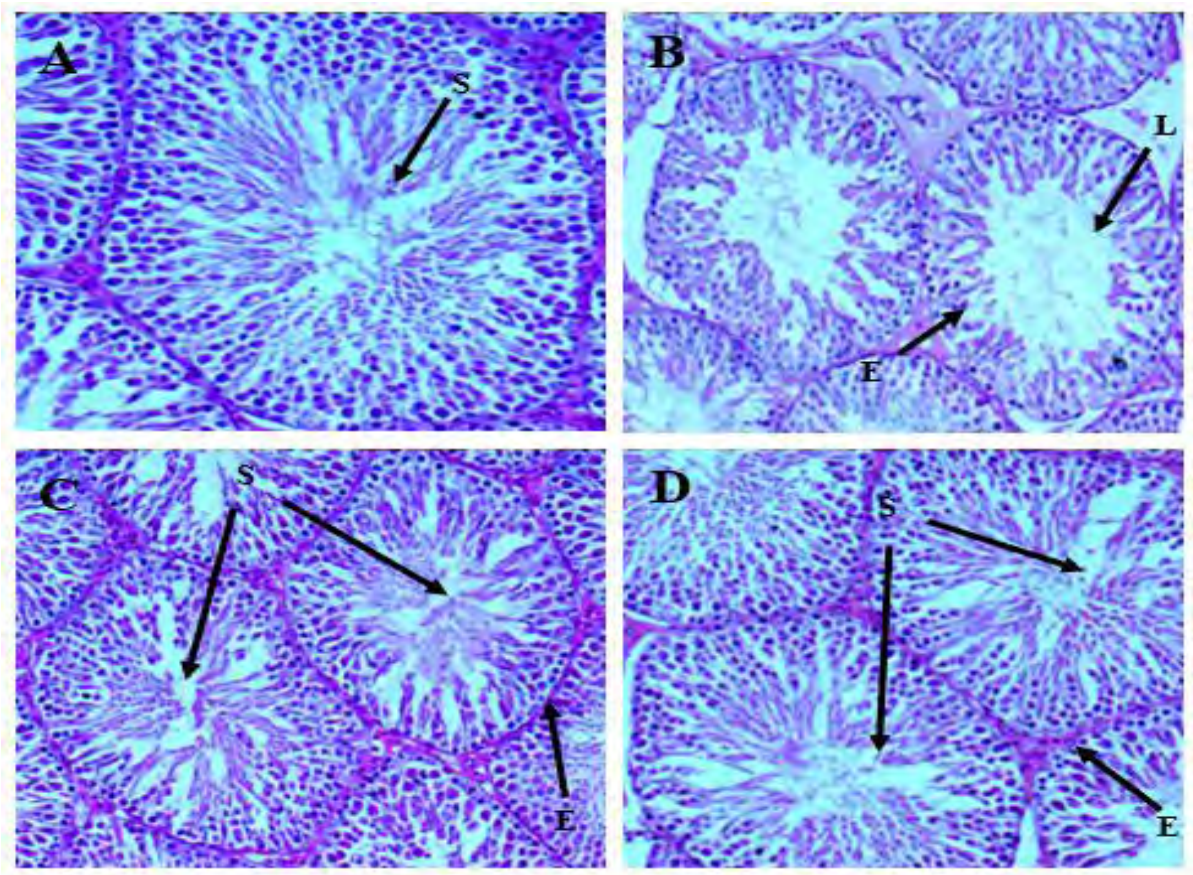


Figure 14: Photomicrograph (40X) of the seminiferous tubules of male rats was shown above in figures as Control(A), Furan(B), Vit C (C) and Vit C+ F(D) respectively. The first photomicrograph (A) shows that the lumen was filled with mature spermatids having arranged tubules with normal germ cells. In photomicrograph (B), the 30mg dose of furan caused decrease in number of spermatids causing changes in arrangement of tubules and degenerated epithelial with an empty lumen. Photomicrograph of group Vit C compared to control group was normal morphology of germ cells and lumen are filled with the spermatids. The group D have almost same morphology as compared to control group facing slight changes in epithelial morphology and slightly a smaller number of spermatids in the lumen. Lumen (L), Spermatozoa (S), Epithelium (E).

Table 5: Means ± SEM After 28 days, histopathological parameters of caput epididymal tissues were determined in control and treated adult male rats

Parameters	Control	Furan	Vit C	Vit C +F	P-Value
Tubular diameter µm	438.44±16.14	432.74±8.15	437.35±15.76	434.74±34.53	P=0.9906
Lumen diameter µm	415..40±.03	401.19±14.66	411.33±31.88	407.04±21.69	P=0.9413
Epithelial height µm	66.21 ± 4.04	58.78±2.69	63.28±2.96	62.91 ± 5.45	P=0.3743

The values are stated as Mean $\pm$ SEM. *, **, ***, **** showing significant variance at $p < 0.05, p < 0.01, p < 0.001$ and $p < 0.0001$ respectively.

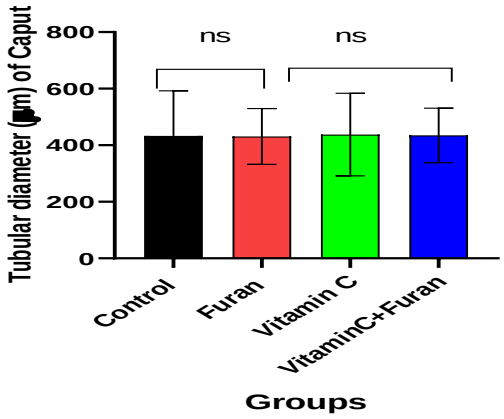


Figure 15: Examining the Tubular Diameter of caput by one-way ANOVA test, it was demonstrated that the tubule diameter in furan treated group was reduced as compared to that of control group. The test was non-significant as p-value was higher than 0.05 i.e., (p >0.05).

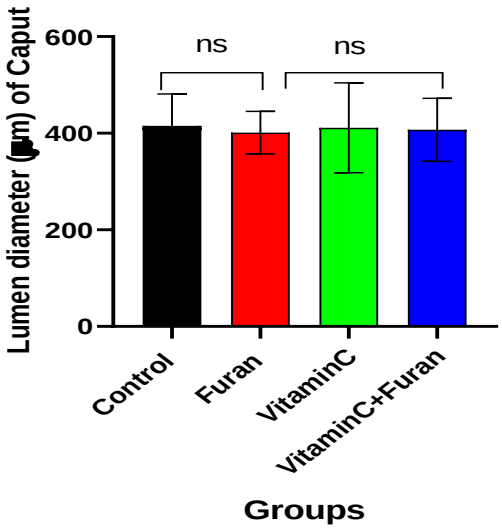


Figure 16: The Lumen Diameter of Caput was determined between the control and treatment groups using one-way ANOVA followed by Tukey's test. It was demonstrated that there was an increase in the lumen diameter of caput in furan-treated group compared to the control group, the p value was strongly significant as (p=0.9413)

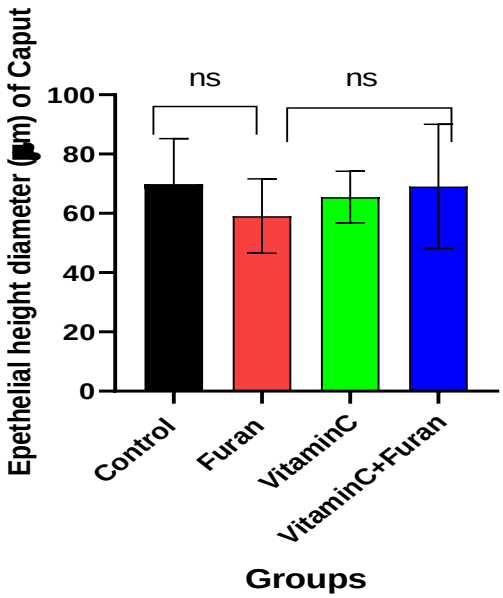


Figure 17: The epithelial height of caput was determined by one-way ANOVA test followed by Tukey's test also known as post HOC test. The test results revealed that there was a substantial decrease in epithelial height of furan- treated group compared to control group, Vit C group and Vit C+ F group. Comparing the furan treated group with that of control group the test was highly significant as (****p<0.0332). On comparing the Vit C and Vit C+ F group it was seen that the test was not significant as p-value was greater than 0.05.

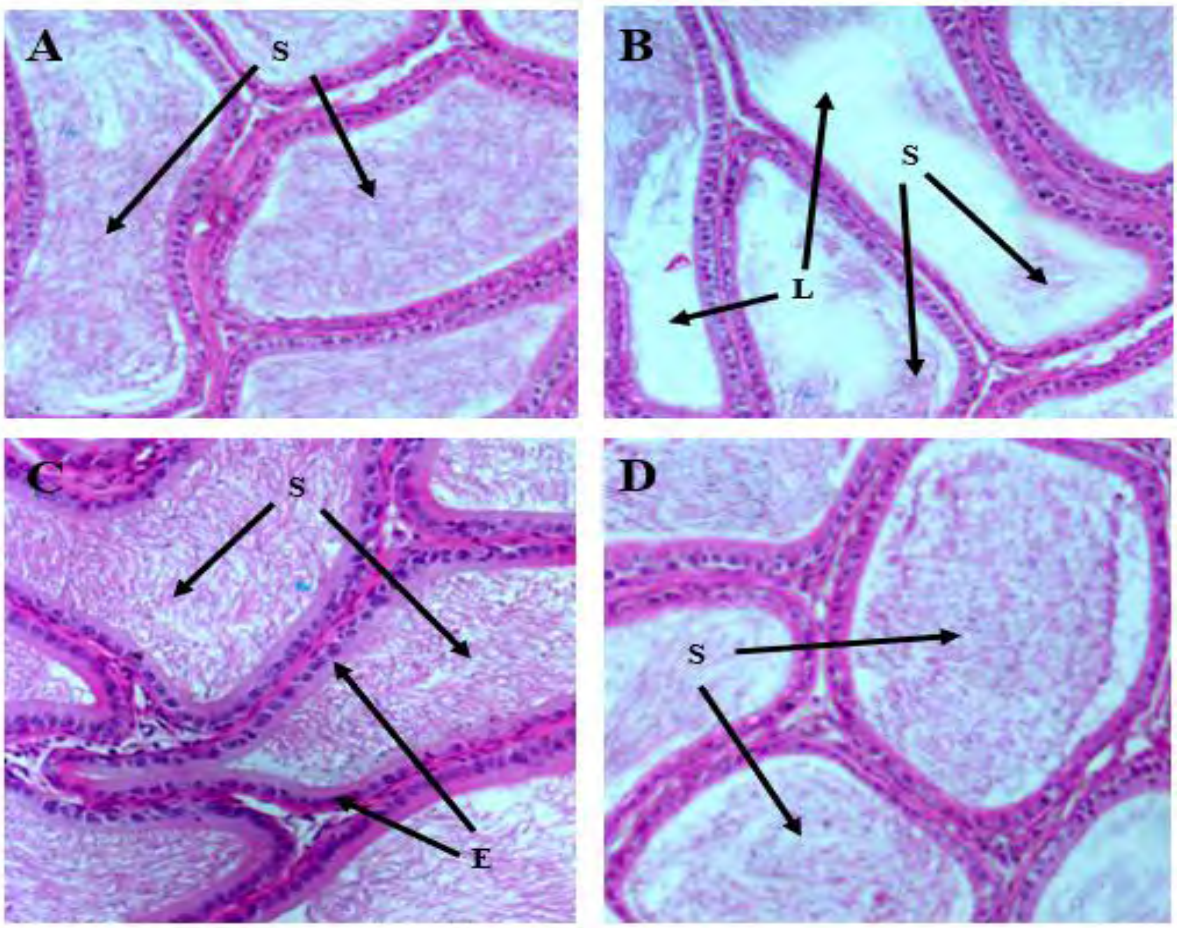


Figure 18: Photomicrograph of the caput epididymis (40X) of male rats was shown above in figures as Control (A), Furan (B), Vit C (C) and Vit C+ F (D) respectively. The first photomicrograph (A) shows that the lumen was filled with the spermatids having arranged tubules with normal germ cells. In photomicrograph (B), the dose of furan caused decrease in number of spermatids causing changes in arrangement of tubules and empty lumen. The sperm concentration increased at one point causing decrease in overall sperm concentration. Photomicrograph of group C as compared to control group has normal morphology of germ cells and lumen are filled with the spermatids due to the dose of Vit C. The group D have almost same morphology as compared to control group facing slight changes in epithelial morphology. Spermatozoa (S), Lumen (L), Epithelium (E).

Table 6: Means ± SEM Histopathological parameters of caudal epididymal tissues in control and treated male adult rats after 28 days of treatment.

Parameters	Control	Furan	Vit C	Vit C+F	p-value
Tubular diameter (µm)	547.60±17.19	531.28±14.16	543.88±12.80	536.32±11.40	P=0.8441
Lumen diameter(µm)	449.61±13.37	454.71±9.28	446.80±12.06	448.84±22.73	P=0.9856
Epithelial Height(µm)	56.14 ± 3.14	41.77± 2.50[a*]	53.45 ± 2.56	47.03±4.54	P=0.0162

The values are stated as Mean $\pm$ SEM. *, **, ***, **** showing significant variance at $p < 0.05, p < 0.01, p < 0.001$ and $p < 0.0001$ respectively.

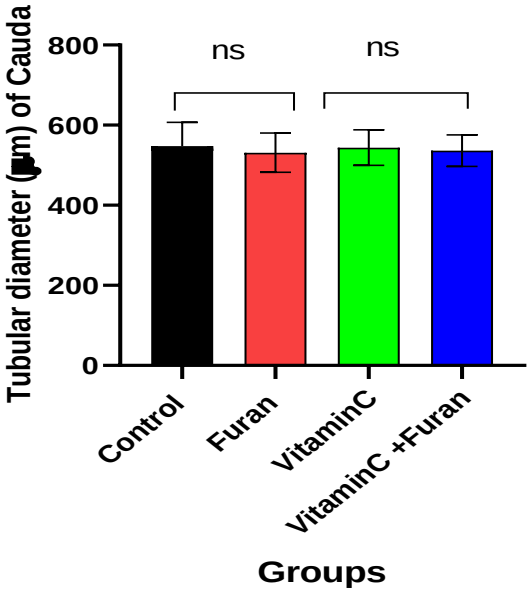


Figure 19: Tubular Diameter of cauda was examined and then compared by using one-way ANOVA in combination with Tukey's test. The tubular diameter in furan treated group was reduced as compared to control group. The test was non-significant as (p= 0.84410). The test was not significant among control group and Vit C treated group. The test among furan and furan + Vit C group showed significance as (*p=0.0377).

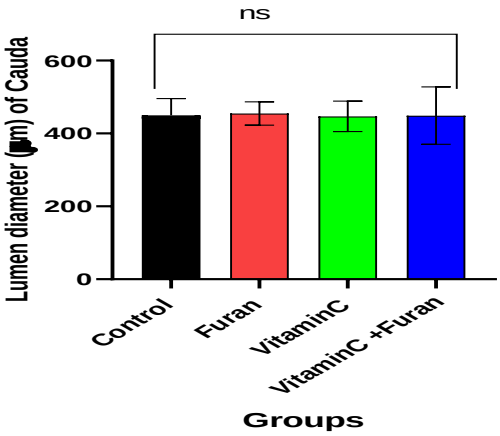


Figure 20: The lumen diameter of furan treated group as compared to control group was no significance difference. However, there was an increase in lumen diameter of cauda but non-significant as (P=0.9856)

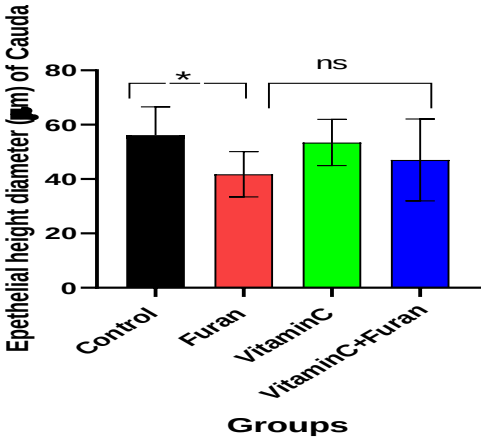


Figure 21: Epithelial height of cauda was examined and then compared by using one-way ANOVA in combination with Tukey's test. The epithelial height in furan treated group was reduced as compared to control group. The test was significant as (****p<0.0001). The test was not significant among control group and Vit C treated group. The test among furan and furan + Vit C group showed significance as (**p=0.0062).

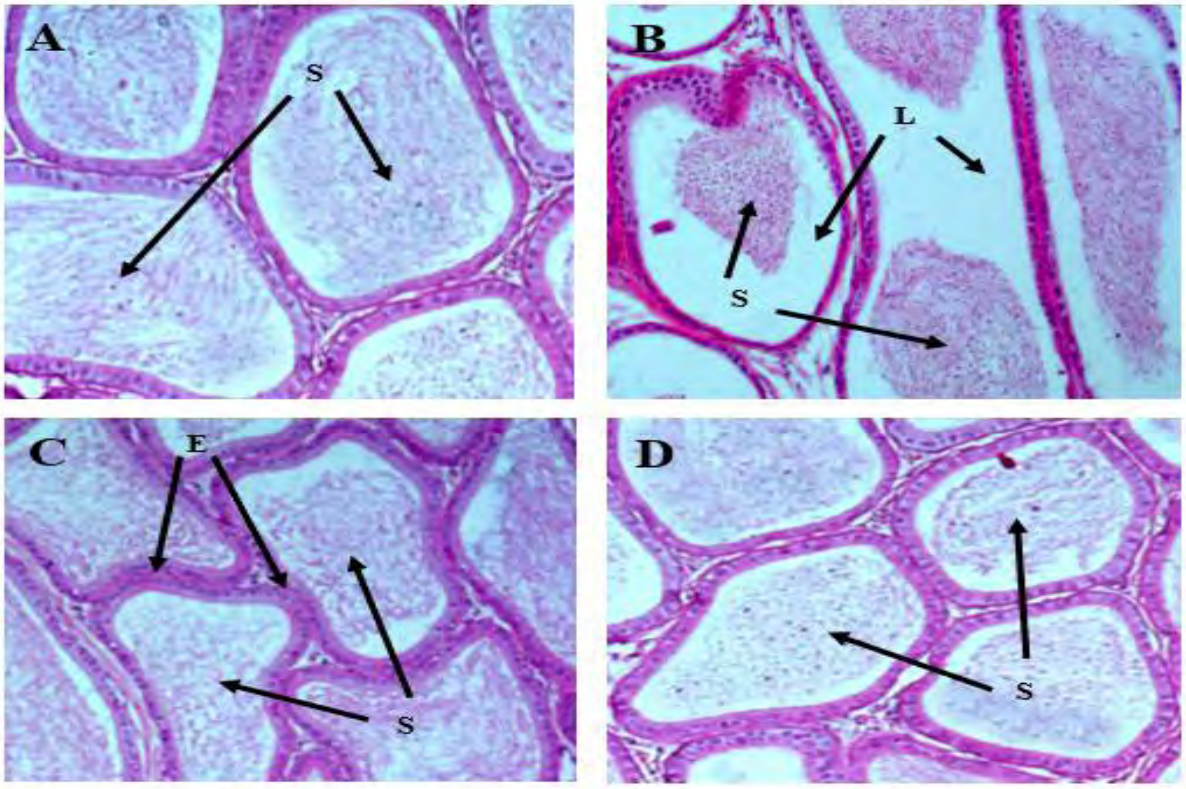


Figure 22: Photomicrograph of the cauda epididymis (40X) of male rats was shown above in figures as Control (A), Furan (B), Vit C (C) and Vit C+ F (D) respectively. The first photomicrograph (A) shows that the lumen was filled with the spermatids having arranged tubules with normal germ cells. In photomicrograph (B), the dose of furan caused decrease in number of spermatids causing changes in arrangement of tubules and empty lumen. The sperm concentration increased at one point causing decrease in overall sperm concentration. Photomicrograph of group Vit C as compared to control group was observed normal morphology of germ cells and lumen are filled with the spermatids due to the dose of Vit C. The group Vit C + F was showed the same morphology as compared to control group facing slight changes in epithelial morphology. Spermatozoa (S), Lumen (L), Epithelium (E).

Hormonal Analysis:

The concentration of testosterone was significantly reduced ($p<0.01$) in furan group as compared to control. No significant change in testosterone levels was observed in Vit C and Vit C+ Furan as compared to control. Least significance ($p=0.03$) was determined between furan and Vit C group. No significant change was observed in T levels when comparison was made among Furan vs Vit C+Furan and Vit C vs Vit C + Furan groups.

Table 7: Mean± SEM of testosterone (ng/dl) concentration after 28 days of treatment in control and treated adult male rats were determined.

Groups	Control	**Furan**	**Vit C**	**Vit C+F**	**P value**
Testosterone (ng ml^{-1})	8.02± 0.16	5.79±0.39^{a**}	7.46±0.80^{b*}	6.65±0.10	P<0.0097

The values are stated as Mean $\pm$ SEM. *, **, ***, **** showing significant variance at $p < 0.05, p < 0.01, p < 0.001$ and $p < 0.0001$ respectively.

The above-mentioned table shows that the testosterone hormone was decreased in furan treated group. The p-value was less than 0.05 making the test significant (***p<0.0097).

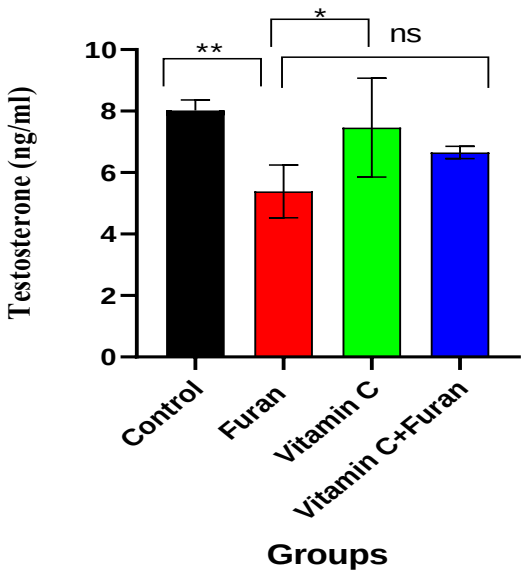


Figure 23: Comparison of plasma testosterone levels between control, furan treated, Vit C and Vit C+ F groups. One-way ANOVA and Tukey's test showed that testosterone levels were lower in the furan-treated group than in the control group (**p 0.0097)

Discussion

Furan is a dangerous chemical that has a variety of negative effects on living animals' biological systems. Scientists are paying more attention to furan because of its endocrine disrupting properties and carcinogenic potential (Karacaoglu and Selmanoglu, 2010; Pandir, 2015a). Furan inhibits reproduction by interfering with spermatogenesis, resulting in the death of germ cell lining and Leydig cells and effects reproductive function of male rats (Cooke *et al*., 2014; Rehman *et al*., 2019). In a report exposure to furan an increased level of furans is seen in the ejaculation of infertile males in comparison to fertile donors in screening survey (Galimova *et al*., 2015). Orally administering of 4 mg/kg/day furan for 90 days with 5 days per week caused anoxidative damage in testis of rats (El-Akabawy and El-Sherif (2016). Vitamin C (Vit C) is a water soluble anti-oxidant that breaks up chains, helps to decrease oxidative stress resulted by chemicals and the environment (Ambali *et al*., 2018). Ascorbic acid is an antioxidant that humans do not produce, so it must be obtained through the diet (Fernandes *et al*., 2011). Vit C can decrease unstable species of Sulphur, oxygen, and radicals of nitrogen in supplement to regenerating additional antioxidants in body, such as alpha tocopherol (Vit E). In human plasma, Vit C has also been observed to be effective in inhibiting lipid peroxidation caused by radicals of peroxide (Sunil *et al*., 2017).

The accessory organ such as testicular weight also observed that there is non-significant difference then control, previous report also showed there is no significant difference in the body weight and testicular weight according to the (Gill *et al*., 2010). The weight of testis and epididymis did not show any significant difference after the exposure to furan as designed early (Karacaoğlu and Selmanoğlu, 2010). All the biochemical and morphological alterations were found in furan treated rats' group. Significant reduction occurred in the antioxidant such as CAT, SOD and POD While noticeable increases occur in oxidative stress markers, ROS and TBARS were elevated. Tissue histology

demonstrated alteration in cellular morphology. The hormonal analysis test also showed that in the furan intoxicated rats reduced the hormonal level.

In our present finding rats exposed to furan for 28 days caused significant decrease in POD, CAT, and SOD in the testicular plasma in as compared to control group. Furan exposure reduce the level of antioxidants while the LPO and ROS level increases, resulting in oxidative damage in the testes, epididymis, and hypothalamus of the exposed animals (Owumi *et al*., 2020).

The current study also showed that the furan exposure to rats significantly increased the ROS and TBARS level. The ROS and LPO levels were elevated in testicular plasma in furan treatment group (Rehman *et* al., 2019). Furan exposure also cause DNA damage and elevated the ROS level in lymphocytes (Pandir, 2015). Histopathological results of present study indicated decrease in number of spermatids causing changes in arrangement of tubules and empty lumen. The sperm concentration increased at one point causing decrease in overall sperm concentration.

Furan intoxicated rats significantly decreased the testosterone level. The decrease in testosterone level resulted in reduction of the number of germ cells in stages VII and stage VIII of spermatogenesis cycle due to the reduction in the seminiferous epithelium height (Kumar et al., 2006; Karacaoglu and Selmanoglu, 2010). Another study showed that furan exposure significantly diminishes the testosterone level (Rehman *et al*., 2019; Owumi *et al*., 2020).

In the current research, testicular tubular diameter significantly decreased while testicular lumen diameter was significantly increase in comparison with the control group, the result match with previous literature (Karacaoğlu and Selmanoğlu, 2010; Rehman *et al*., 2019). However, Furan treatment groups reduced the epithelial height in comparison to control group. Caput epididymis morphometry showed that the caput tubular diameter significant difference in furan treated group while there are no changes in lumen diameters and caput epithelial heights as compared to control one. In Cauda epididymis

lumen and ductular diameter of cauda showed non-significant changes. Furan treated group significantly reduced the cauda epididymis epithelial height as compared to control (Rehman *et al*., 2019).

The present study showed that rats intoxicated with furan alter the histopathology in the male rats, reproductive organs caput epididymis cauda epididymis and testis. The number of spermatids in the cauda epididymis and caput epididymis decreases due to disruption of tubule arrangement and empty lumen due to furan toxicity, as previously reported in the literature. Furan toxicity decreases spermatids and effects tubular arrangement (Karacaoğlu and Selmanoğlu, 2010; El-Hakim *et al*., 2018; Rehman *et al*., 2019). The epithelium, sperm number, and empty lumen were all affected by the furan in the rats' testis seminiferous tubule histology. Degenerative changes and coagulative necrosis were present, as well as disorganization of the germinal epithelium. Vacuolations within cells, few or no spermatogenic cells, loss of germinal epithelium, and Sertoli cells only in atrophic tubules in the tubules, there were numerous uni and multinucleated giant spermatids, as well as an accumulation of cellular debris. Tubule basement membrane thickening was observed. Interstitial edema and congestion were also visible. In the testicular stroma, hyperplasia of Leydig cells and mild leucocytic infiltrations were found (El-Hakim *et al*., 2018).

In recent study, administration of vitamin C was testified to significantly protect the rats reproductive and accessory organs from furan induced toxicity restoring antioxidants morphological alterations and hormonal level to normal. All rats survived for 28 days of experiment. The present research showed that the Vit C does not affect the rats body weight. the previous literature also showed that Vit C non-significantly increased the body weight (Omshi *et al*., 2018). Vit C has no effect on the weight of reproductive organs such as the testis, epididymis, vas deferens, prostate, and seminal gland (Leite *et al*., 2017). Our results showed that SOD, POD and CAT activities were significantly lower, and ROS and MDA levels were significantly higher in the testis of Furan-treated

rats. It has been also reported that furan exposure elevated the ROS and LPO level and decreases the activity of antioxidants (Rehman *et al*., 2019). Vit C restored the levels of reduced ROS and TBARs and increased CAT, SOD and POD in testis. Vit C scavenge the ROS level and due to the increase level of antioxidant.

SOD is a critical for determining oxidative stress levels (Altan *et al*., 2003). SOD is a cellular antioxidant that catalysis the reaction of free anion superoxide (O_2) radical to produce hydrogen peroxide (H_2O_2) and water (H_2O), which is then degraded by CAT or GPx (Buettner *et al*., 2011). By increasing free radicals, stress can cause electron transport disorders. Free radicals are unpaired electron molecules that are highly reactive and can alter nucleic acids, proteins, and fatty acids in plasma lipoproteins and cell membranes (Nandi *et al*., 2019). MDA is formed when poly-unsaturated fatty acids in cell membranes are easily reduced by free radicals during the process of lipid peroxidation (Min *et al*., 2018). Antioxidant therapy can help to reduce lipid peroxidation. In general, lipid peroxidation can be divided into three stages: initiation, propagation, and termination. Antioxidants transfer hydrogen atoms during the termination stage, lowering the reactive potential of non-radical compounds (Akbarian *et al*., 2016). Furthermore, CAT is a crucial antioxidant enzyme that uses hydrogen peroxide as a substrate and aids in the maintenance of cellular redox homeostasis (Akbarian *et al*., 2016).

It has been described that vitamin C pretreatment protects against carbamazepine-induced low sperm count, owing to vitamin C antioxidant activity, which appears to decrease free radical in the testis, thus improving testosterone and gonadotropin production for spermatogenesis (Akorede *et al*., 2020). Another study discovered that ascorbic acid (AA) reduces oxidative stress in Cd-treated rats' testes. SOD, CAT, and POD levels are restored by vitamin C, while MDA and ROS levels are reduced (Chen *et al*., 2018). Vitamin E increased the levels of the antioxidant enzymes CAT, SOD, and POD, while lowering the levels of TBARS and MDA (Hidayatik *et al*., 2021).

The furan causes a change in the histomorphology of the testicular tissue, but vitamin C restores the histology. The sperm concentration of the testis, caput, and cauda epididymis is retained by keeping testis cauda epididymis diameter, lumen space and caput. In previous studies, Vit C treatment maintained against changes in oxidative changes measuring parameters, sex hormones, sperm quality, relative weight of pituitary and testis, and histological alteration (Akorede *et al*., 2020). Morphometry revealed that furan alters the morphometry of the testis, but vitamin C reversed the effects. Supplementing with ascorbic acid during puberty restored the percentage of normal seminiferous tubules and prevented an increase in acidophilic germ cell frequency in the epithelium (Leite *et al*., 2017). Studies also showed that Vit C recover the seminiferous tubular diameter (Fernandes *et al*., 2011; Rauf *et al*., 2021).

The testosterone level increased in the vitamin c treatment group in this study. Vitamin C increased testosterone levels by lowering the level of ROS (Traber *et al*., 2011; Jahan *et al*., 2019). During the spermiogenesis process, testosterone regulates libido and promotes spermatid elongation (Dutta *et al*., 2017). As a result, stress has a negative impact on feed consumption, body weight, libido, growth rate, and animal production (Lara and Rostagno *et al*., 2013). Another study found that vitamin C increases testosterone levels (Hidayatik *et al*., 2021).

Conclusion

Present study suggests that furan can induce reproductive damage in rats by inducing oxidative stress in testes and sperms. This damage can be successfully ameliorated by using vitamin C. However molecular level studies are needed to understand complete mechanism of these activities.

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