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Glucocorticoid-Induced Dose-Dependent Reproductive Impairments in Male Albino Rat

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Abstract

The present study is aimed at elucidating the effect of different doses of Dexamethasone (DEX) on testis and epididymis of male Wistar rats. Thirty-six rats were divided into six groups, each containing six rats: one control (CON), and five groups of rats treated with five (30 µg, 40 µg, 50 µg, 60 µg, 70 µg/100 g body weight) doses of dexamethasone intraperitoneally (i.p.) for twenty-one days. The results revealed a dose-dependent decrease in testes and epididymis weight. DEX-treated rats evidenced significant increase in TBARS levels, this being the highest in 70 µg/100 g body weight (bw) which could be a consequence of the highest level of free radical generation. The activity level of antioxidative enzymes Superoxide Dismutase (SOD), Catalase (CAT), Glutathione (GSH), and protein content significantly declined in dexamethasone-treated rats in a dose-dependent manner. Histological observation revealed different degrees of germ cell degeneration/alterations in testis and epididymis The present finding suggests that exposure to dexamethasone (70 µg/100 g bw) can potentially lead to severe impairments in male reproductive tissues (testis and epididymis) structure and function, which may consequently lead to male infertility.

Keywords: Dexamethasone, Epididymis, Male Fertility, Seminiferous Tubule, Testis

1. Introduction

Reproduction is one of the most important physiological phenomena and it is concerned with procreation and, hence, perpetuation/survival/sustenance of species. Organs of the male and female reproductive systems play a central role in sexual reproduction by producing, nourishing, and housing the gametes. The human male reproductive system consists of gonads called testis, and a series of ducts (ductus epididymidis, vas deferens, ejaculatory duct, and urethra) that serve to produce, store, process and discharge the spermatozoa. The epididymis is an essential reproductive organ that has many functions like sperm transport, concentration, protection, storage, maturation, and acquisition of motility of sperm. Testicular tissue has a much faster rate of cell division and mitochondrial oxygen consumption than other tissues, as well as relatively higher quantities of unsaturated fatty acids. Due to low oxygen pressure, cells are forced to compete vigorously for oxygen. Therefore, the testicular tissue is vulnerable to oxidative stress which may be directly linked to male infertility[1]. Due to exposure to x-ray, pollutants and chemical substances and, also, varicocele can exacerbate the oxidative stress and induce apoptosis of germ cells and ultimately impair spermatogenesis[1]. Exposure to glucocorticoids leads to a variety of undesired physiological consequences including induction of oxidative stress by enhanced Reactive Oxygen Species (ROS) in many tissues and organs[2]. Oxidative stress serves as a common pathway for several factors to act, and adversely affect male reproductive functions. ROS is produced endogenously through immature spermatozoa and seminal leukocytes and at a normal level it takes part in vital male reproductive functions. Oxidative stress

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is prompted through the production of immoderate ROS that supersedes endogenous antioxidant capacity. Excess ROS initiates a sequence of reactions, disrupting cell organelles and rendering spermatozoa non-functional[3].

Dexamethasone (DEX) is a synthetic glucocorticoid, fluorinated at position 9, is a multitasking molecule that influences almost all physiological processes including reproduction[4] due to their long biological half-life (36 h to 54 h). DEX is particularly used for treating conditions such as inflammation, allergic and autoimmune disorders, leukemia, nausea, and endocrinopathies, which require a prolonged glucocorticoid support[5,6]. Numerous studies have been conducted on DEX for short periods, but very few have been conducted on DEX over long periods in a dose-dependent manner. Therefore, the present study, aimed to clarify the effects of increasing doses of DEX (30 µg, 40 µg, 50 µg, 60 µg, and 70 µg/100 g body weight) to male Wistar rats for a total of twenty-one consecutive days, on the testis and epididymis.

2. Materials and Methods

All experimental procedures were performed in accordance with the national and international guidelines and regulations, and were approved by the Institutional Animal Ethics Committee (IAEC) of SLT Institute of Pharmaceutical Sciences, Guru Ghasidas Vishwavidyalaya, Bilaspur (Registration Number: 994/GO/Re/S/06/CPCSEA; ReferenceNo.10/IAEC/Pharmacy/2021).

2.1 Animal Procurement and Maintenance

The male Wistar rats were obtained from Defence Research and Development Establishment (DRDE), Gwalior, India. Experiments were performed on rats aged 3-4 months and weighing 180 ± 10 g. Animals were housed in plastic cages and maintained at room temperature (25 ± 1 °C) with free access to a standard pellet feed and tap water. They were exposed to the natural day-night cycle (~12 h/12 h) and kept under standard laboratory conditions. After an acclimatization period of two weeks, rats were randomly divided into six groups.

2.2 Chemicals and Reagents

All the chemicals including Dexamethasone (DEX), 1,1,3,3-Tetra-Ethoxy Propane (TEP), Glutathione reductase (GSH), reduced glutathione and reduced nicotinamide adenine Nucleotide Phosphate (NADPH), Butylatedhydroxytoluen (BHT), Thiobarbituric Acid (TBA), Phenazinemethosulphate (PMS), Nitro Blue Tetrazolium (NBT), Folin's Reagent, Bovine Serum Albumin (BSA) and picric acid were purchased from Central Drug House (CDH), New Delhi, India.

2.3 Experimental Design

The rats were randomly assigned to six groups, each containing six animals (n = 6) and treated as follows:

Groups	**I**	**II**	**III**	**IV**	**V**	**VI**	**Duration**
DEX Dose/100 g body weight/day	Normal saline	30 **µg**	40 **µg**	50 **µg**	60 **µg**	70 **µg**	21 days

2.4 Drugs and Treatment

DEX was first dissolved in a few drops of absolute ethanol and then the working concentration was prepared as desired from the stock by diluting in 0.9% normal saline and injected ip[7,8]. The volume of DEX injected daily was 0.5 mL. Injections were given between 10 and 11 am for twenty-one days.

2.5 Tissue Collection and Processing

After completion of the experiment rats of each group were sacrificed under deep ether anesthesia. Bilateral testis and epididymis were removed, blotted to dry, and weighed. Organs on the right side were washed with normal chilled saline, and photographed for the morphological study. Testis was fixed in Bouin's solution. Organs on the left-side were collected and washed with normal chilled saline, and the tissues were processed for biochemical studies (LPO, SOD, CAT, GSH, and protein).

2.6 Parameters

2.6.1 Histological Preparation

The right testis and epididymis from each animal were fixed in Bouin's fluid. After 2-8 h, the tissues were transferred to a small beaker and placed under running water for 24 h to remove the excess picric acid. Thereafter completion the tissue was kept in 70% alcohol until used. The testis and epididymis were cut vertically into two halves and embedded in paraffin wax and serially sectioned at 5 µm thickness. Paraffin sections were deparaffinized in xylene, rehydrated through decreasing grades of ethanol, stained in Hematoxylin and Eosin [H&E] and then mounted in DPX resin. Sections were observed and photographed in *fluorescence microscope (Axioscope-5, Carl-Zeiss, Germany)*.

2.6.2 Biochemical Parameters

2.6.2.1 Determination of Protein

Protein estimation was done by modified Lowry's Method[9]. Fresh tissue was taken and homogenized. The homogenate was mixed with 0.2 mL of 10% TCA and centrifuged at 2000 rpm for 15 minutes. 1 mL of NaOH (0.2N) was added to the supernatant. To 0.1 mL of the above solution, 0.4 mL of distilled water was added. This protein extract was used to determine the protein. The color developed on addition of Folin-phenol reagent was read in a UV-Vis spectrophotometer at 625 nm.

2.6.2.2 Lipid Peroxidation Assay

Lipid Peroxidation (LPO) was measured by estimation of TBARS level following the modified method of Ohkawa *et al*[10]. Briefly, 10% homogenate was obtained in Tris-Hydrochloride (HCl) buffer (20 mM, pH 7.4). Equal amounts of homogenate and BHT were taken and 3 mL of phosphoric acid and 1mL of TBA were mixed with it. The reaction mixture was heated at 95°C in the water bath for 45 min, and then cooled at room temperature and centrifuged at 3000 rpm for 15 min. The optical density of the supernatant was determined in a UV-Vis spectrophotometer at 535 nm. Lipid peroxidation was expressed in nmol TBARS formed/mg protein.

2.6.2.3 Estimation of Superoxide Dismutase (SOD) Activity

Formation of superoxide radicals was used to determine SOD activity adopting the modified Kakkar *et al*.,[11] method. The tissue was homogenized in 1 mL of Phosphate Buffered Saline (PBS, pH-7.4) and centrifuged at 1500 rpm for 10 minutes to collect the supernatant. The supernatant was mixed with 1.2 mL of sodium pyrophosphate buffer, 0.1 mL of PMS (phenazine methosulphate), and 0.3 mL of NBT (nitro-blue tetrazolium). To the above solution 0.2 mL of NADH was added, followed by 1 mL of glacial acetic acid. Finally, the absorbance was measured at 560 nm.

2.6.2.4 Estimation of Catalase Activity

Catalase (CAT) activity was measured following the modified method of Beers and Sizer[12]. Phosphate buffer (pH-8.2), 2.5 mL, was added to 0.1 mL of the homogenate and centrifuged at 3000 rpm. To the supernatant (40µL) 3 mL of H_2O_2 phosphate buffer was added, mixed thoroughly and the absorbance was measured at 240 nm. The activity of CAT was expressed as the amount of H_2O_2 degraded per minute.

2.6.2.5 Reduced Glutathione (GSH) Assay

Reduced Glutathione (GSH) was quantified by its reaction with 5,5'-Dithiobis (2-nitrobenzoic acid) which leads to the production of a yellow-colored product, following the modified method of Sedlak and Lindsay[13]. Fresh tissue was homogenized in sucrose buffer and centrifuged at 3000 rpm. To 1.5 mL of the supernatant 1.5 mL Tris buffer (pH-8.2) and 0.1 mL DTNB were added. The volume was made up to 10 mL by adding methanol and vortexed. The sample solution was incubated at room temperature for 30 min and then centrifuged at 3000 rpm. The absorbance was measured at 412 nm.

2.7 Analysis of Data

Data were subjected to calculation of means and standard errors. Differences between means of control and experimental data were subjected to statistical analysis adopting Tukey test; *p* values less than 0.05 were considered significant.

3. Results

3.1 Body and Organ Weight

The dose-dependent effect of DEX on the body and organ weights (testis and epididymis) of rats was recorded. The rats treated with the low doses showed mild reduction in the body (Figure 1) and organ (testis and epididymis) weights (Figure 2) in comparison with the high-dose treated groups. The maximum reduction in body and

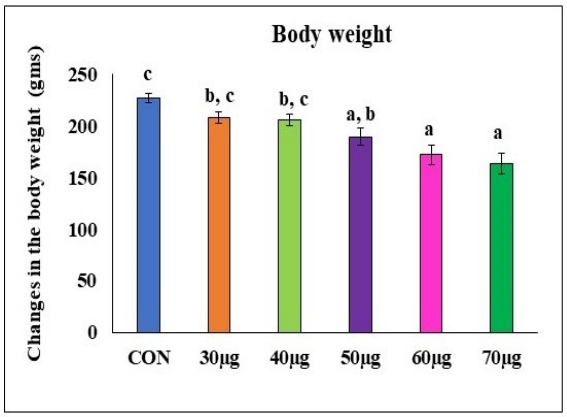

Figure 1. Effect of DEX (30 µg, 40 µg, 50 µg, 60 µg, 70 µg/100 g bw) on body weight of male rats. Graph represents mean±SE, N = 6; CON, Control. The bars bearing the same superscript do not differ significantly (Tukey test, $p< 0.05$).

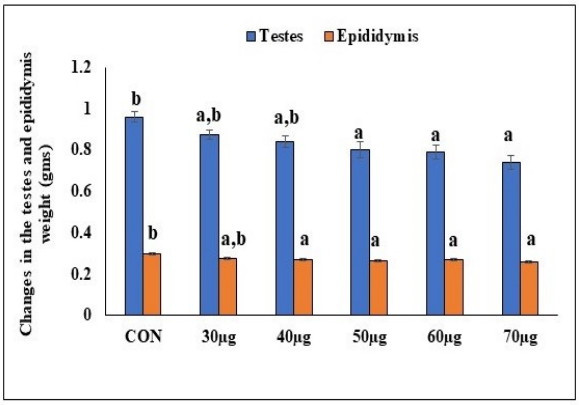

Figure 2. Effect of DEX on weight of testis and epididymis. Graph represents Mean±SE, N = 6; CON, Control. The bars bearing the same superscript do not differ significantly (Tukey test, $p< 0.05$).

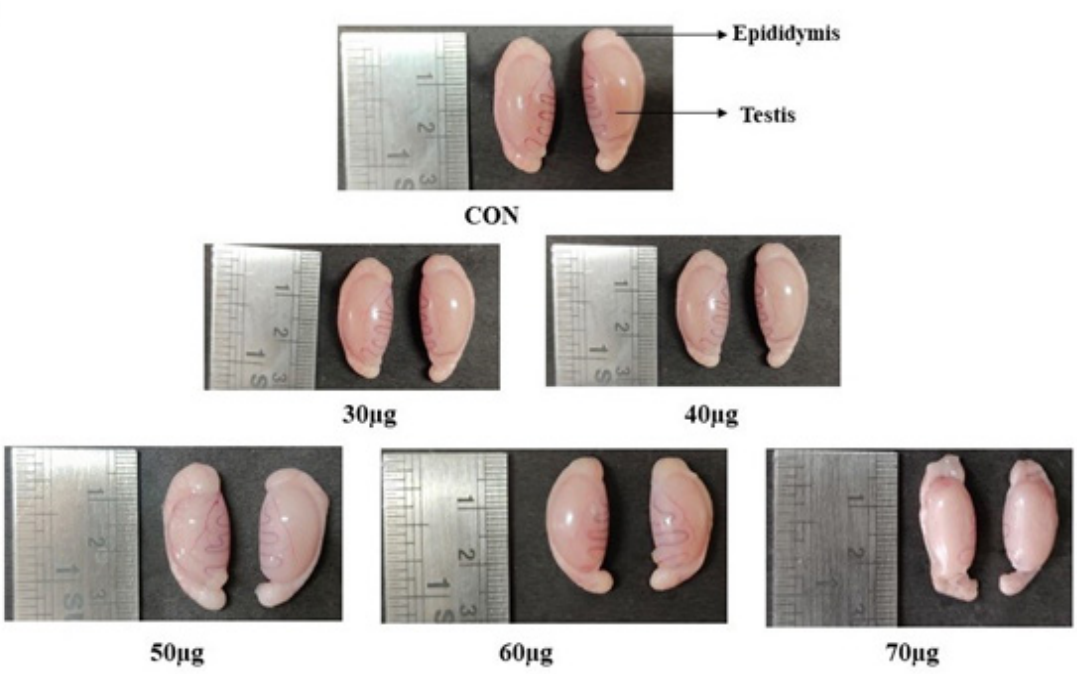

Figure 3. Size of testis and epididymis. Significant difference was observed in testis and epididymis size. The noticeable change in morphology started with 50 µg/100 g bw of DEX group and further shrinkage was found in 70 µg/100 g bw treated group.

organ weights was found in the highest dose group, 70 µg/100 g bw.

3.2 Morphological Changes

Morphological changes were assessed following the treatment of DEX. Low dose DEX (30 and 40 µg/100g bw)-treated groups of rats did not show any remarkable change in the morphology of testis (Figure 3) and epididymis (Figure 4). However, a gradual increase in tissue shrinkage was found in the 50-70 µg/100g bw treatment groups, with the highest shrinkage in the dose groups 60 and 70 µg/100 g bw.

3.3 Histological Changes

Testis sections were observed in *fluorescence microscope (Axioscope-5, Carl-Zeiss, Germany)* and digital images were obtained in a digital camera attached to the microscope. Histological observations of rats in the control group showed normal histoarchitecture of seminiferous tubules**,** epithelium, and active spermatogenesis of testis and cellularity of the epididymis (caput). DEX-treated groups of animals exhibited deterioration of seminiferous tubules, characterized by vacuolation, indistinct seminiferous epithelium, and different degrees of germ cell pathologies in the testis (Figure 5; upper panel-100x, lower panel-200x) and epididymis (caput) (Figure 6; upper panel-100x, lower panel-200x) in a dose-dependent manner wherein treatment of DEX at 70 µg/100 g bw impacted the testicular and epididymal cellularity very severely.

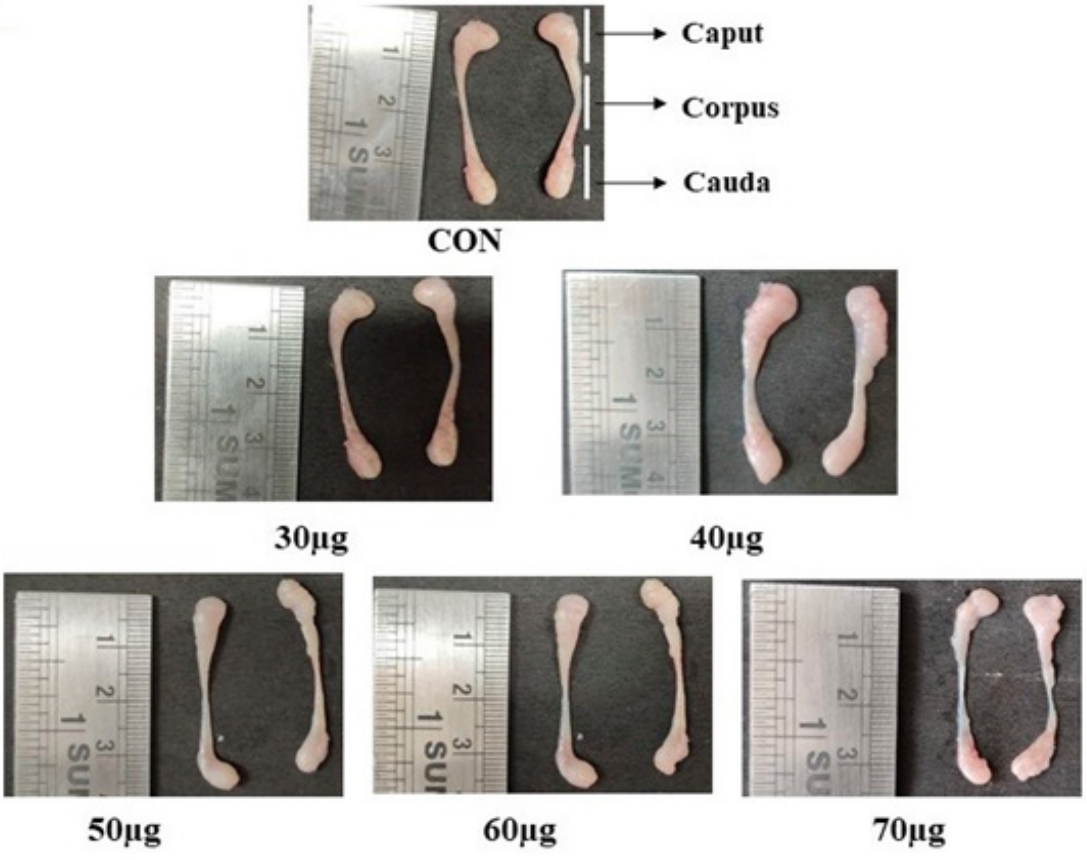

Figure 4. Size of epididymis. The change in size of the epididymis started showing up in 50 µg/100 g bw DEX group and further gradual shrinkage was noticed with the highest deterioration in 70 µg/100 g bw DEX group. The bars bearing the same superscript do not differ significantly.

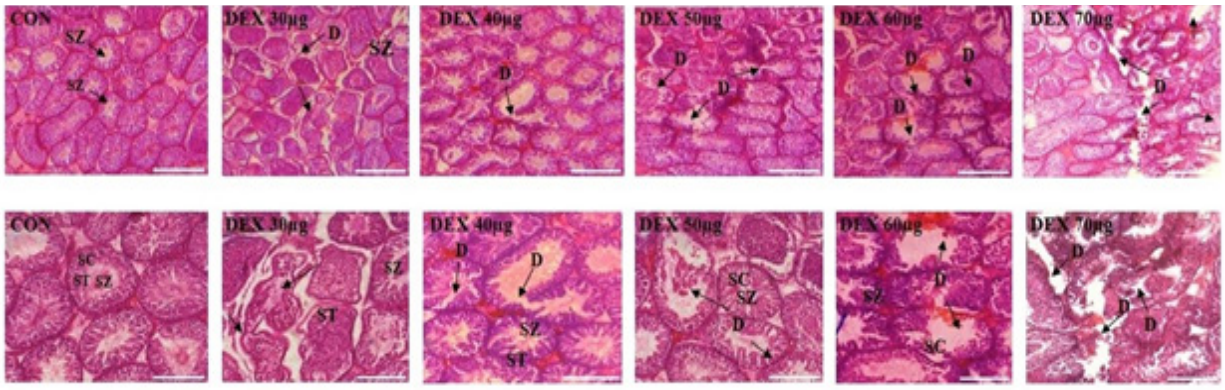

Figure 5. TS of testis**.** In the control group, normal histological structure of seminiferous tubules showing the complete spermatogenic sequence in control rats' testis [spermatocytes: SP; spermatids: ST; spermatozoa about to be released to the lumen: SZ]. Testis of DEX-treated (30 µg, 40 µg, 50 µg, 60 µg and 70 µg/100 g bw) rat shows marked dose-dependent degeneration, the maximum being with 60 µg and 70 µg/100 g bw. DEX-treated rat testis shows marked Degeneration (D) of seminiferous tubules and lack of spermatogenic series. The arrow indicates mild disintegration of the seminiferous tubules with loss of spermatids and spermatozoa, degeneration (arrow) of most spermatocytes and spermatids in the testes was maximum in 60 µg and 70 µg/100 g bw of DEX groups. Magnification of upper panel-100X, lower panel-200X; Scale bar: upper panel 5 µm, lower panel 2 µm.
CON= Control, DEX= Dexamethasone, SZ= Spermatozoa SC= Spermatocytes, ST= Spermatids, D=Degeneration

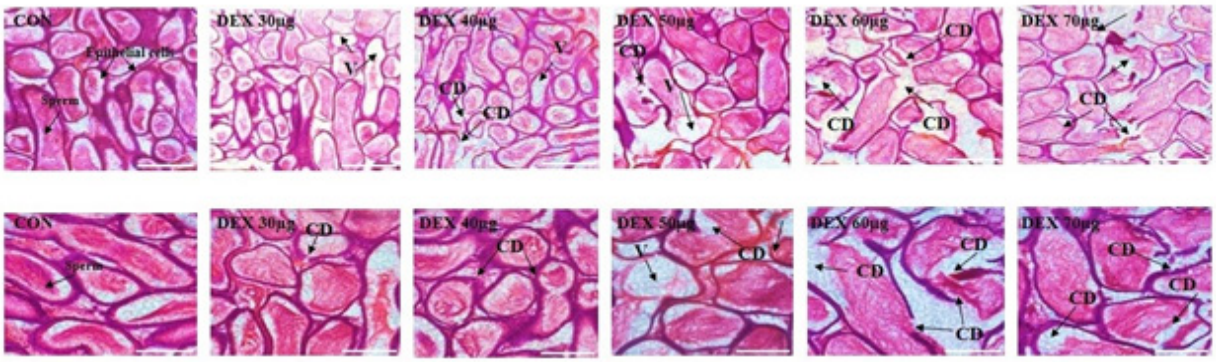

Figure 6. TS of epididymis (caput). Photomicrographs showing alterations in the tissue of the caput epididymis where CON, Control; DEX, Dexamethasone. CON group of rat's shows normal sperm density in the epididymal cells and do not show any Cellular Damage (CD), while as DEX-treated rats showed Vacuole formation (V) with most of epididymal ductules were free from spermatozoa and cellular damages in 70 µg/100 g bw group. Magnification of upper panel-100X, lower panel-200X; Scale bar: upper panel 5 µm, lower panel 2 µm.
CON= Control, DEX= Dexamethasone, V= Vacuole, CD= Cellular Damage

3.4 Markers of Oxidative Stress and Tissue Biochemical Status

The different doses of DEX on their physiological/ biochemical expression resulted in production of free radicals. These free radicals elevated the rate of lipid peroxidation in the testis and epididymis. Increased TBARS levels directly indicated the excessive free radical generation, elevation of oxidative stress, this being the highest in 70 µg/100 g bw group (Figure 7).

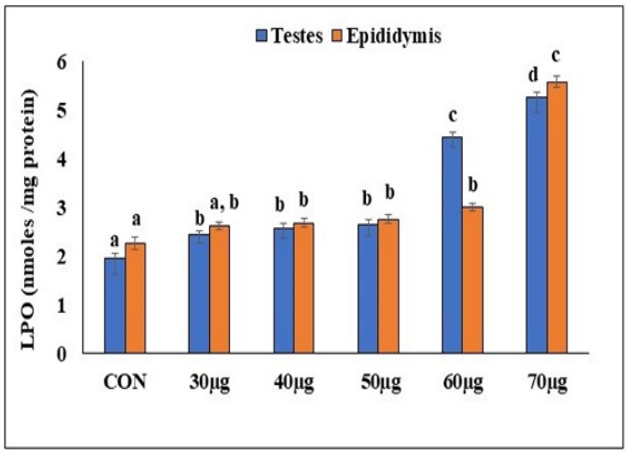

Figure 7. Effect of DEX (30 µg, 40 µg, 50 µg, 60 µg, 70 µg/100 g bw) on TBARS level of testis and epididymis in male rats. Graph represents Mean±SE, N = 6, CON, Control. The bars bearing the same superscript do not differ significantly (Tukey test, $p<0.05$).

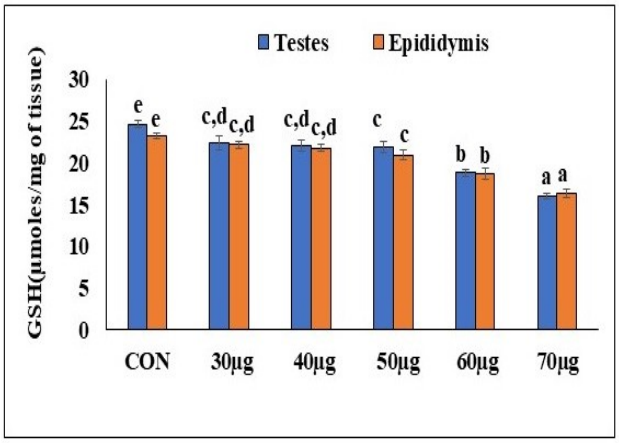

Figure 9. Effect of DEX (30 µg, 40 µg, 50 µg, 60 µg, 70 µg/100 g bw) on GSH activity of testis and epididymis in male rats. Graph represents Mean±SE, N = 6, CON, Control. The bars bearing the same superscript do not differ significantly (Tukey test, $p < 0.05$).

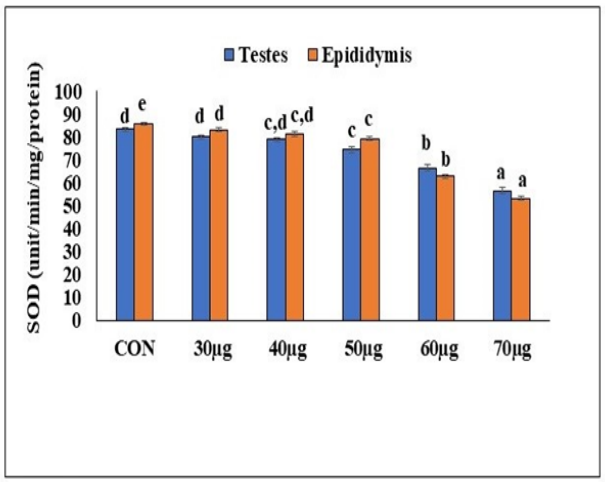

Figure 8. Effect of DEX (30 µg, 40 µg, 50 µg, 60 µg, 70 µg/100 g bw) on SOD activity of testis and epididymis in male rats. Graph represents Mean ± SE, N = 6, CON, Control. The bars bearing the same superscript do not differ significantly (Tukey test, $p<0.05$).

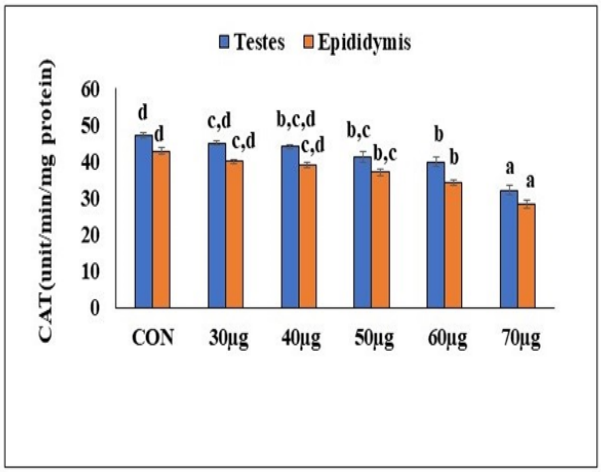

Figure 10. Effect of DEX (30 µg, 40 µg, 50 µg, 60 µg, 70 µg/100 g bw) on CAT activity of testis and epididymis in male rats. Graph represents Mean±SE, N = 6, CON, Control. The bars bearing the same superscript do not differ significantly (Tukey test, $p<0.05$).

3.5 Antioxidant Status

The activity of superoxide dismutase (Figure 8), Glutathione (GSH) (Figure 9), and Catalase (CAT) (Figure 10) significantly declined in the DEX-treated group with the most decline in the 70 µg/100 g bw DEX-treated animals. Total cellular protein content was found significantly decreased in the DEX-treated groups, and of the maximum decrease was found in 70 µg/100 g bw treated animals (Figure 11).

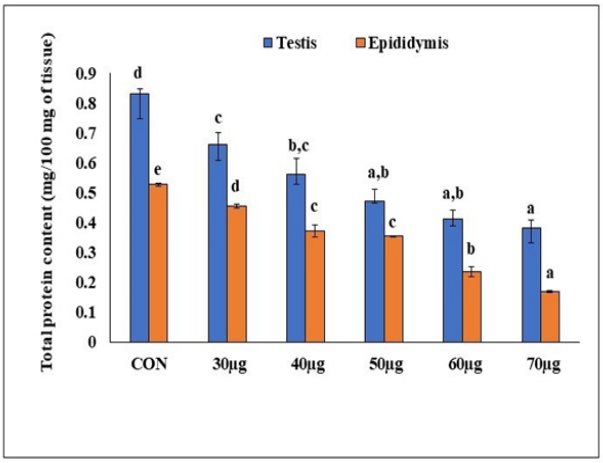

Figure 11. Effect of DEX (30 µg, 40 µg, 50 µg, 60 µg, 70 µg/100 g bw) on total protein content of testis and epididymis. Graph represents Mean±SE, N = 6, CON, Control. The bars bearing the same superscript do not differ significantly (Tukey test, $p<0.05$).

4. Discussion

The present study highlights oxidative stress as a potent mediator of DEX-induced testicular and epididymal damage in male Wistar rats by impairing antioxidant defense mechanisms. The results of the present study, considered in the line of the existing literature, suggest that long-term exposure to DEX leads to various undesirable physiological consequences due to enhanced production of Reactive Oxygen Species (ROS)[2,15]. Stress-induced levels of glucocorticoids result in several pathologies including profound reproductive and immune dysfunctions[4,16]. The mechanism of DEX-induced germ cell apoptosis is not yet fully understood, and the present study suggests a link between ROS and DEX-induced germ cell apoptosis. The present study establishes the involvement of DEX in testicular oxidative stress at two highest doses (60 µg/100 g bw and 70 µg/100 g bw). The present investigation, which is in line with Page *et al.*,[17] found that the body weight of male Wistar rats treated with DEX decreased significantly. Gravimetric analysis showed tissue specific variation, where testis and epididymis were found more affected in 60 µg/100 and 70 µg/100 g bw. This signifies that there might be any alteration in cellular architecture causing atrophy leading to decrease in organ weight. Varying degrees of degenerative changes have also been reported in animals treated either with toxic chemicals[18] or endocrine disruptors[19]. Results reveal a dose-dependent gradual increase in tissue shrinkage with the exposure to DEX which has a deleterious effect on testis and epididymis.

Histological evidence from the present study reveals a variety of damages to the reproductive tissues, mild disintegration and degeneration of seminiferous tubules with loss of spermatids and spermatozoa, which were most prevalent in the 60 and 70 µg/100 g body weight DEX-treated animals. This is consistent with the findings from earlier studies[20-22] which reported that glucocorticoids suppress testicular steroidogenesis through diverse mechanisms at the hypothalamus and pituitary gland and direct actions on Leydig cells (action on P450scc, 3HSD, and P450c17), respectively. The report suggests testicular apoptosis as well as Leydig's cell impairments following administration of glucocorticoids in a dose-dependent manner[23-25].

Oxidative stress is caused by a disruption in the steady-state relationship between ROS production and the body's antioxidant defense capacity. ROS is continuously produced by active metabolic processes under normal physiological conditions. Tissues have developed defense mechanisms against ROS by having an elaborate array of antioxidant enzymes and free radical scavengers, and testis are not an exception in this regard[3]. A series of studies point out oxidative stress as a major factor for male infertility[26,27] since ROS has the potential to disrupt androgen-producing Leydig cells and cause increased lipid peroxidation and DNA fragmentation in germ cells. Antioxidant enzymes are the first line of defense against the harmful effects of Reactive Oxygen Species (ROS)[14]. SOD catalyzes the dismutation of superoxide radicals to Hydrogen Peroxide (H_2O_2) and molecular Oxygen (O_2), whereas catalase and GSH are known to be responsible for H_2O_2 detoxification[28,29]. In the present study, DEX-treated animals had significantly lower activity of testicular SOD, CAT, and GSH which resulted in significantly higher lipid peroxidation in the testis. One of the most common processes caused by oxidative stress is lipid peroxidation. Increased ROS levels are thought to be a possible indicator of apoptosis[30], which can be prevented by a variety of antioxidants[31]. This phenomenon is indicated in the present study. A low concentration of DEX will cause less stress, whereas a high dose of DEX causes more stress on the testis and epididymis. This increased ROS production and low antioxidant enzyme

levels in the testis and epididymis treated with 70 µg/100 g body weight may be signs of this increased stress. It is possible for an antioxidant molecule to overcome DEX suppression in healthy persons.

4. Conclusion

In conclusion, DEX treatment resulted in a dose-dependent impact on biochemical parameters and tissue features of the testis and epididymis as judged by the increased free radical load, down regulated antioxidant enzymes, and scanty cellularity in terms of different degrees of germ cell degeneration as compared to the control group of rats showing normal histoarchitecture of the seminiferous tubule, epithelium and active spermatogenesis. These testicular and epididymal damages were noted to be more severe and at higher levels in rats that received exogenous DEX supplementation at a dose of 70 µg/100 g body weight. These damages may have been caused by glucocorticoid-induced stress that would decrease the production of gonadotropin, which in turn would affect the hypophyseal-testicular axis and possibly impaired reproductive functions. It is therefore suggested that long-term use of synthetic glucocorticoids such as DEX, may potentially lead to male infertility.

5. Acknowledgments

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