Effect of naproxen on testicular morphometry and serum gonadotropin level of cadmium intoxication in rats

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SUMMARY

Nonsteroidal anti-inflammatory drugs that are cyclooxygenase (COX) enzyme inhibitors like naproxen (NAP) and prostaglandins play important roles in the regulation of reproductive functions in females. Cadmium (CAD) is a toxicant that poses effects on various organs in humans and experimental animals. The aim of this study is to investigate the ameliorating effect of naproxen (NAP) against cadmium-induced testicular toxicity on adult Wistar rats with the objectives on histology, hormonal and biochemical parameters. The total number of animals used were fifteen (15) and were grouped into three (3) (n=5 per group): control (CON); Cadmium (CAD; 5 mg/kg) and Cadmium + Naproxen (NAP; 20 mg/kg) for the period of thirty days. Twenty-four hours after the last administration, the animals were weighed and sacrificed using chloroform as a sedative. The organs were located, removed and weighed using an electronic sensitive analytical balance (Sartorius). Results: There are significant differences in the lumen of the control and the group exposed to CAD, which shows that CAD has the ability to disrupt the seminiferous tubules. Significant differences were also found in the epithelium thickness of the CAD-treated groups—with or without NAP.

Morphological alterations were not reversed in the rats exposed to CAD treatment with NAP. Hormonal findings shows that Luteinizing hormone (LH), Follicle stimulating hormone (FSH) were markedly significant ($p<0.05$), but biochemical findings were not significant. In conclusion, CAD induced testicular damage as seen by the histological and morphological observation of the testes, and naproxen ameliorated cadmium-induced testicular damage.

Key words: Cadmium – Naproxen – Testis – Morphology – Gonadotropin – Testicular damage – Heavy metals – Non-steroidal anti-inflammatory drug (NSAID)

INTRODUCTION

Cadmium (CAD) is a toxic heavy metal extremely harmful to humans and other mammalian species.

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Submitted: July 28, 2020. **Accepted:** March 29, 2021

It is present in air, soil, sediments, water and smoke. After intake, CAD accumulates in multiple organs and tissues (Järup and Akesson, 2009). Exposure to CAD leads to a wide range of health defects (Nemmiche et al., 2007; Donpunha et al., 2011). For this reason, the International Agency for Research on Cancer has categorized CAD as carcinogenic to humans and animals (IARC, 1993). Unfortunately, human exposure to cadmium is on the increase, particularly in developing countries, due to rapidly growing industries with increasing consumption and subsequent release into the environment. Humans are exposed to CAD, mainly through occupational and environmental contamination (Satarug and Moore, 2004). Non-occupational exposure to CAD predominantly results from smoking, air pollution and consumption of CAD-contaminated seafood and water (Järup et al., 2000; Waisberg et al., 2003). Cadmium has an extremely long biological half-life of 15 years that essentially makes it a cumulative toxin in the liver and kidney (Ercal et al., 2001). However, the dose above which CAD causes early health effects is largely unknown (Thijssen et al., 2007). Several underlying mechanisms at cellular or even molecular levels have been proposed for CAD toxicity. There is a growing interest in the plausible manners of protection from adverse effects induced by CAD exposure. Hence, it seems reasonable that special attention should be directed to agents that can prevent or reduce this metal-induced toxicity (Brzoska et al., 2015; de Moura and Ribeiro, 2015).

Naproxen (NAP) is a Nonsteroidal Anti-inflammatory Drug (NSAID) of the propionic acid class and is commonly used for relief in a wide variety of conditions in which there is pain, fever, swelling and stiffness. It is the preferred NSAID for long-term use in people with a high risk of cardiovascular complications (Richy et al., 2004). Naproxen works by reversibly inhibiting both the Cyclooxygenase-1 (COX-1) and Cyclooxygenase -2 (COX-2) enzymes (Duggan et al., 2010; Hinz et al., 2008). Using agents that have not exhibited many side effects for both treatments and protective measures and that have strong anti-inflammatory effects is very important. Therefore, this study aims at investigating the acute effects of naproxen on cadmium-induced testicular damage.

MATERIALS AND METHODS

Breeding of the Animals

The study was conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, approved by the Institutional Review Board of the University of Ilorin with code UERC/BMS/097, and every effort was made to minimize both the number of animals used and their suffering. Male Wistar rats (8-10 weeks old) were obtained from the animal holding of the Department of Anatomy, Ladoke Akintola University of Technology, Ogbomoso (Oyo State, Nigeria). The rats were housed in cages (made from wood, wire gauze and net) under natural light and dark cycles at room temperature. The floor of the cages was made with wood to make it comfortable for the rats, and it was covered with sawdust to provide a soft floor for the rats and to make cleaning of the cage convenient when littered. They were fed with rat pellets purchased from approved stores, and water was given *ad libitum.* They were grouped and left to acclimatize for 2 weeks before the study commenced.

Experimental design

The total numbers of animals used were fifteen. They were grouped into three: one (1) control and two (2) experimental groups with consideration towards size variation. Cadmium was added to drinking water, while naproxen was administered using a feeding tube (orogastric cannula, size-6). The animals were treated for a period of thirty days.

Group 1: (control) (CON): (n = 5): Given rat pellets and distilled water.

Group 2: (n = 5): Given cadmium-exposed (CAD) and pellets.

Group 3: (n = 5): Given cadmium + naproxen – treated rats (CAD+NAP) and pellets.

The CAD-treated rats received 5 mg/kg of cadmium as reported previously (Shagirtha et al., 2011).

Cadmium-exposed rats were treated with Naproxen at 20 mg/kg body weight for 15 days. Naproxen was administered orally through orogastric cannula.

Collection and processing of the samples

Twenty-four hours after the last administration, the animals were weighed and thereafter sacrificed by the use of chloroform as a sedative. Blood was collected by cardiac puncture into EDTA bottles for serum FSH, LH and C-reactive protein (CRP) analysis. The animals' abdominal cavity was opened by a midline abdominal incision and the reproductive organs (Testes) were removed. Testes were fixed in Bouin's fluid. Epididymis were placed in 0.9% normal saline and minced with 0.9% of normal saline for semen analysis. Sperm morphological characteristics were graded as normal or otherwise, according to Saalu et al. (2008).

Biochemical Studies

The level of LH, FSH and CRP were estimated using Accu Bind ELISA Microwell by Mono bind Inc. Lake Forest, CA 92630, USA. Malodialdehyde levels in serum were measured according to the method of Stock and Domandy (1971).

Luteinizing Assay Procedure

LH was quantitatively determined according to the manufacturer instruction based on the method of Wennink et al. (1990). Essentially, biotinylated monoclonal and enzyme-labeled antibodies are directed against LH epitope— the immunologically active site. The reaction between LH antibodies and native LH forms a sandwich complex that binds with the streptavidin coated to the well. Following the completion of the required incubation period, the enzyme-LH antibody bound conjugate is separated from unbound enzyme-LH conjugated by separation and decantation. The activity in the antibody-bound fraction is directly proportional to the native antigen concentration. The activity of the enzyme present on the surface of the well was quantified by reaction with suitable substrate to produce color.

Twenty-five microliters (25 µl) of the standard, the specimens and controls were dispensed into appropriate wells. Twenty-five microliters (25 µl) of enzyme conjugate reagent were pipetted into the wells and thoroughly mixed for 30 seconds, and incubated at a temperature of 36°C for 60 min. The microtiter wells were rinsed and flicked 5 times with 300 µl of washing solution. The wells were struck sharply with absorbent paper to remove all residual water. Hundred microliters (100 µl) of TMB substrate solution were added to each well, and mixed and then incubated at room temperature for 15 min. The reaction was stopped using 100 µl of stopping solution. The samples were gently mixed for 30 seconds until the blue color changed to yellow. Absorbance was read at 450 nm with Rayto: RT-2100C, Microplate Reader within 15 min.

Follicle Stimulating Hormone Assay Procedure

Follicle stimulating hormone was assayed according to Simoni et al. (1997). 25 µl of the standard, the specimens and controls was dispensed into appropriate wells. 50 µl of enzyme conjugate reagent was pipetted into the wells and thoroughly mixed for 30 seconds and incubated at a temperature of 36°C for 60min. The microtiter wells were rinsed and flicked 5 times with 300µl of washing solution. The wells were struck sharply with absorbent paper to remove all residual water. 100µl of TMB substrate solution were added to each well and mixed and then incubated at room temperature for 15 min. The reaction was stopped using 100µl of stopping solution. The samples were gently mixed for 30 seconds until the blue color changed to yellow. Absorbance was read at 450 nm with a microtiter well reader within 15 min C-reactive protein (CRP).

The level of plasma CRP was estimated by using commercially available enzyme-linked immunosorbent assay (ELISA) kit (Accu Bind ELISA Microwell by Monobind Inc. Lake Forest, CA 92630, USA). The test was performed using the previously described method of López-Alcaraz (2014). Briefly, 0.050ml (50 µl) of the appropriate serum reference, control or specimen was pipetted into assigned well. 100 µl of CRP-Enzyme Reagent was added to all wells. The microplate was swirled gently for 20-30 seconds for proper mixing.

The plate was incubated at room temperature for 15 min. The content of the microplate was discarded by decantation (using absorbent paper to blot the plate dry). 350 µl of buffer were added

and decanted (the procedure was done thrice for proper washing of microplate). 100µl of working substrate were added to all wells. The reagents were added in the same order to minimize reaction time differences between wells. The plate was incubated at room temperature for 15 min. 50 µl of stop solution were added to each well and was mixed for 15-20 sec. Finally, each absorbent was read at 450 nm in a microplate reader (using a well referenced wavelength of 620-630 nm to minimize well imperfection).

Determination of Plasma Malondialdehyde

Malondialdehyde levels in plasma were measured according to the protocol outlined by Stocks and Domandy (1971). The reaction mixture contained 100 µl of plasma and 20% trichloroacetic acid (1 ml). The above were mixed and centrifuged at 2000 rpm for 5 min to obtain the supernatant. 0.5 ml of supernatant is mixed with 0.7% thiobarbituric acid (1 ml); the tubes were heated in a water bath at 100^{o}C for 20 min and all tubes were cooled in water. The spectrophotometer was blanked using the reagent blank at 532 nm. Absorbance of tests and standards were read. Malondialdehyde level was calculated in plasma.

Semen Analysis

After sacrifice, the caudal epididymis of rats in each of the experimental group was removed and minced thoroughly in a specimen bottle containing 0.9% normal saline for a few min to allow the sperms to become motile and swim out from the caudal epididymis (Saalu et al., 2008).

Sperm count and motility

After 5 min incubation at 37ºC (with 5% CO_2), the semen was then taken with a 1 ml pipette, dropped on a clean slide, and covered with cover slips. The slides were examined under light microscope for sperm motility (Saalu et al., 2008) and with the aid of the improved Neubauer hemocytometer (Deep1/10mm LABART, Germany) counting chamber, as described by Pant and Srivastava (2003); the spermatozoa were counted under the light microscope. The counting was carried out in five thoma chambers.

Sperm morphology

This was done as described by Saalu et al. (2013). The sperm morphology was evaluated with the aid of a light microscope at x400 magnification. The caudal sperm was taken from the original dilution for motility, and diluted 1:20 with 10% neutral buffered formalin (Sigma- Aldrich, Canada). In wet preparations using phase contrast optics, the spermatozoa were categorized. In this study a spermatozoon was considered morphologically normal and abnormal based on:

Well-defined acrosomal region comprising 40-70% of the head area.

No neck, mid-piece or tail defects and no cytoplasm droplet more than one-third the size of normal sperm head.

Histological Procedures

The histology of the testes was done by modifying the method reported by Bancroft and Gamble (2008). The organs were harvested and fixed in Bouin's fluid for 24h, after which it was transferred to 70% alcohol for dehydration. The tissues were washed through 90% and absolute alcohol and xylene for different durations before they were transferred into two changes of molten paraffin wax for 1 hour each in an oven at 65ºC for infiltration. They were subsequently embedded, and serial sections were cut using rotary microtome at 5 micrometers.

The tissues were picked up with albumenized slides and left to dry on a hot plate for 2 min. The slides were dewaxed with xylene and washed with absolute alcohol (2 changes); 70% alcohol, 50% alcohol and then water for 5 min. The slides were then stained with Hematoxylin and Eosin. The slides were mounted in DPX. Photomicrographs were taken at x100 magnification.

Statistical analysis

Results obtained from the analysis of andrological parameters as well as testicular body weight ratio of the Wistar rats were analyzed statistically to see the correlation between the results using Sigma plot (SPW v. 12). The results were presented as mean ± SEM with significant level at P-value <0.05 while the histological

examination, Sperm analysis, FSH and LH were carefully studied and analyzed to establish any correlation between the groups.

RESULTS

Naproxen reversed spermatid defects in cadmium-induced testicular damage. Exposure of the rats to cadmium led to significant decrease in the normal concentration of spermatids, which was however reversed in the rats treated with naproxen (Fig. 1A). There was also increase in the percentage of head defects in the cadmium-exposed rats, which was reversed in the naproxen – treated rats (Fig. 1B). There was increased percentage of neck and tail defects respectively in the rats exposed to cadmium. Whereas there was no change in this in the rats treated with naproxen, the defects were not aggravated (Fig. 1 C, D).

Naproxen ameliorated sperm motility defect in cadmium-induced testicular damage. Cadmium exposure led to significant decrease in sperm concentration, motility and motility percentage, which were however reversed in the rats treated with naproxen (Fig. 2 A-C).

Naproxen exacerbated testicular damage in cadmium-exposed rat. Enlargement of interstitial space and disruption of seminiferous tubules were observable in the cadmium-exposed group in contrast to the control group. Treatment with naproxen did not reverse the morphological damage caused by cadmium; rather, more morphological damage was seen, as there was enlargement in space of the lumen of the tubule (Fig. 3). H&E (×100). The arrow shows point of degeneration of basement membrane. IS- Interstitial Space. LT- Lumen of the tubule.

Naproxen reversed reduction in luteinizing hormone caused by exposure of rats to Cadmium. Although there was no significant change in the C-reactive protein (CRP), malondialdehyde (MDA) and follicular stimulating hormone (FSH) in the rats exposed to cadmium, the luteinizing hormone (LH) was significantly reduced. However, the reduction in LH in cadmium exposed rats was reversed with naproxen treatment (Table 1).

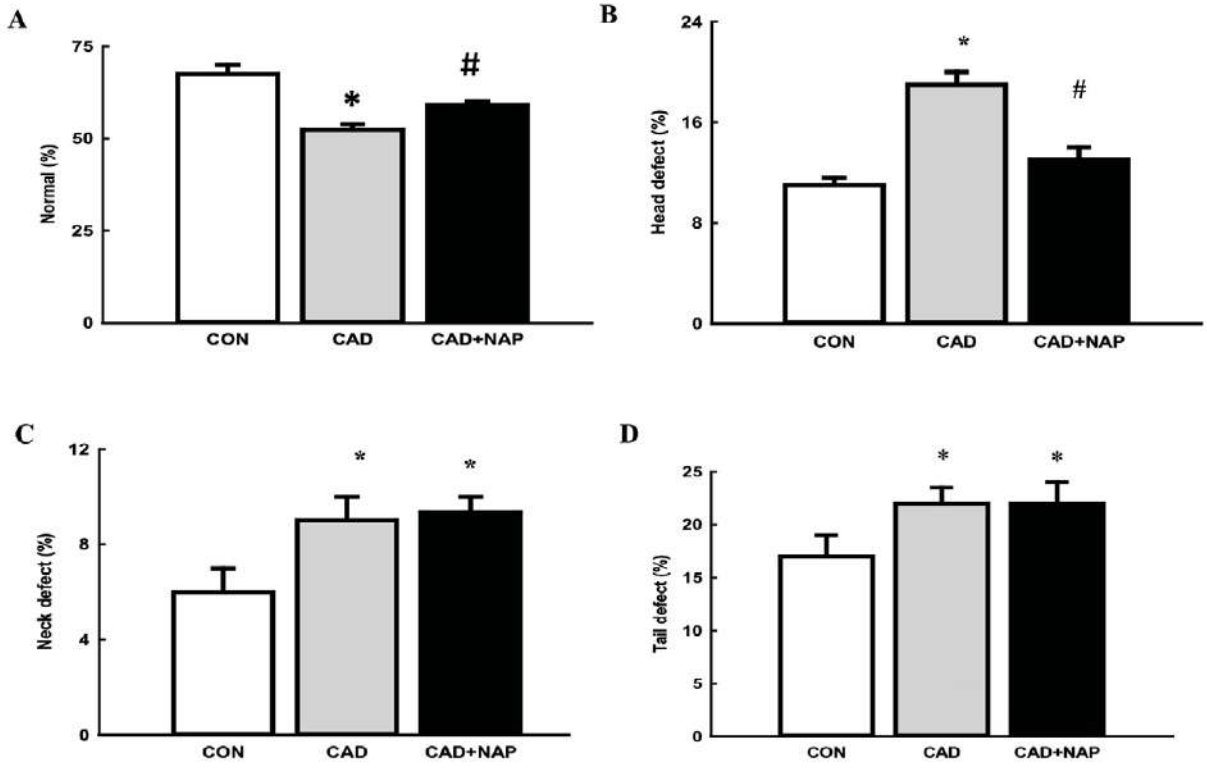

Fig. 1.- Naproxen reversed spermatid defects in cadmium-induced testicular damage. % normal spermatid (A), % head defect (B), % neck defect (C), % tail defect (D). (*p<0.05 vs. control; #p<0.05 vs. CAD).

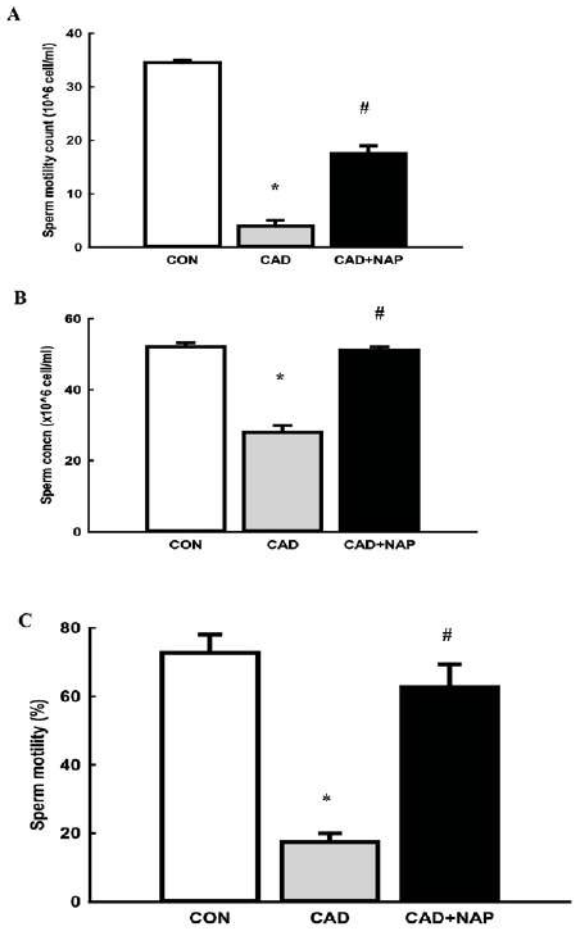

Fig. 2.- Naproxen ameliorated sperm motility defect in cadmium-induced testicular damage. Sperm motility (A), Sperm concentration (B) and % sperm motility (C). (*p<0.05 vs. control; #p<0.05 vs. CAD).

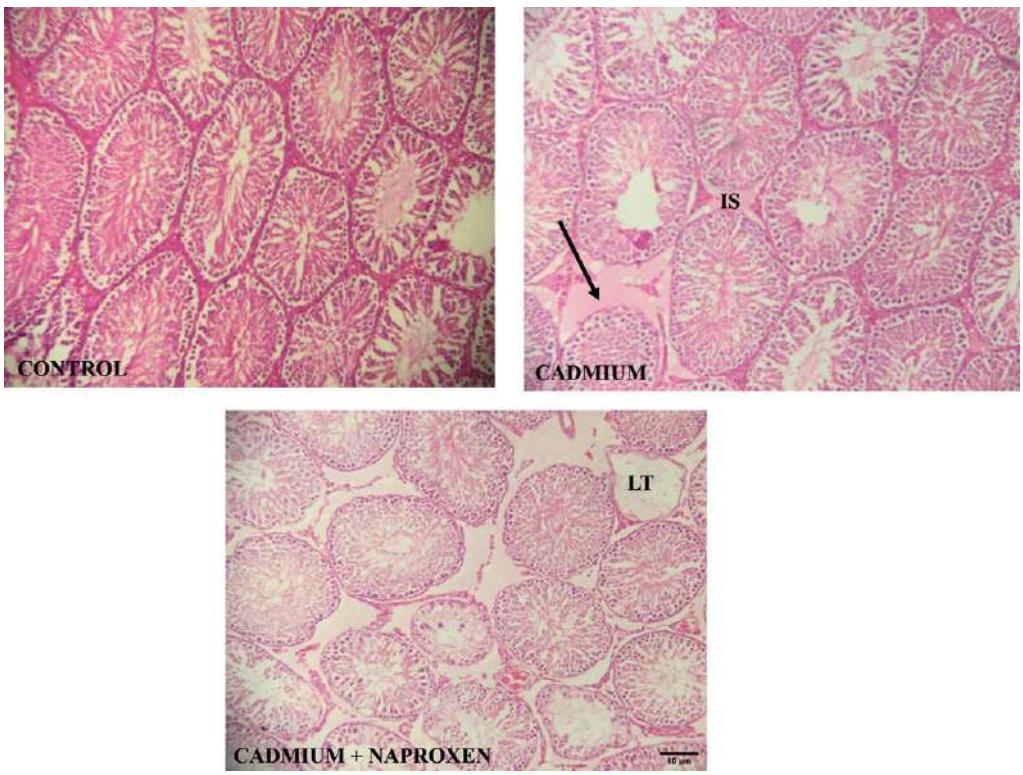

Fig. 3- Naproxen exacerbated testicular damage in cadmium exposed rat. Photomicrographs of testis of the control, cadmium exposed and naproxen treated rat. H&E (100X, scale bar = 10 µm). Arrow shows point of degeneration of basement membrane. IS- Interstitial Space. LT- Lumen of the tubule.

Table 1. Naproxen reversed reduction in luteinizing hormone caused by exposure of rats to Cadmium.

	Con	CAD	CAP+NAP
CRP (mg/dl)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± .0
MDA (mg/dl)	2.7 ± 0.2	2.8 ± 0.1	2.7 ± 0.2
FSH (mg/dl)	3.6 ± 0.6	4.6 ± 0.6	3.3 ± 0.6
LH (mg/dl)	4.9 ± 0.2	2.4 ± 0.2*	3.6 ± 0.3#

CRP- C-reactive protein, MDA- Malondialdehyde, FSH- Follicular stimulating hormone, LH- Luteinizing hormone. * Significant difference from control, # significant difference from CAD.

DISCUSSION

In the last decades, discussion regarding the relationship or development of diseases induced by environmental pollutants is increasing in the scientific literature. Among the numerous toxic substances to which human and other mammalian species are exposed to is cadmium, a heavy metal whose half-life ranges between 10 and 30 years. It promotes extensive damage to several tissues such as liver, kidney, lungs and blood (Nair et al., 2013). Testes and spleen are affected by acute cadmium (CAD) intoxication (Santos et al., 2004; Yiin et al., 2000).

The result of this study showed that CAD caused atrophy and disruption of the seminiferous tubules, increase in interstitial space with reduced Leydig cells, disruption of germinal layers, degeneration and disorganization of cellular layer of the seminiferous tubules, disruption of interstitial tissue, reduced spermatocyte and reduction of mature spermatids. This observation is in agreement with previous studies (Martins et al., 2007; Adamkovicova et al., 2010).

Histopathological damage to the seminiferous tubules may be as a result of unique vasculature and morphological layout of the blood testes barrier (BTB) of the testis (Erica et al., 2009). Treatment of cadmium-exposed rats with NAP does not reverse the morphological alterations.

It was also observed that CAD exposure caused reduction in total sperm count, sperm concentration, sperm motility, and luteinizing hormone. Reduction of follicle stimulating hormone, luteinizing hormone and Leydig cell can probably be the reason for the release of immature sperms into the lumen, reduced sperm count, sperm concentration and sperm motility, which can lead to male infertility. A previous study has reported altered circulation of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in CAD exposure (Lafuente et al., 2004). The endocrine disruption induced by CAD is likely to be multi- factorial mediated via its effects on Leydig cells and/or the hypothalamic-pituitary-testicular axis (Erica et al., 2009). NAP treatment however reversed these defects.

The morphological observations of the testis showed that CAD causes malformation to spermatids. Animals treated with CAD have significantly increased malformed spermatids compared to those of the CON. NAP treatment however reversed this defect. Therefore, it cannot be concluded that naproxen can be teratogenic to spermatogenesis.

Exposure to CAD does not lead to inflammation as found in this experiment. There were no significant differences found in the level of CRP in serum of the control group and both groups exposed to CAD, this is in disagreement with previous reports by Kumas et al. (2016) who recorded increase in serum CRP level of rats treated with CAD (0.5 mg/kg/day) intraperitoneally for 10 days and Ajilore et al. (2016) where treatment of rats with 5 mg/kg of cadmium through oral route for 2 weeks led to significant reduction in hepatoglobin concentration a CRP. Administration of CAD does not increase level of MDA in serum as there were no significant differences found between the CON group and those exposed to CAD. This result did not correlate with another report by Majedah et al. (2010) who observed increase in serum level of MDA leading to oxidative stress after intraperitoneal administration of a single dose of cadmium at 5 mg.

It was discovered in this study that NAP could cause damage to the seminiferous tubules, as there was increased lumen enlargement and reduction of spermatogonium compared to group that was exposed to CAD without treatment. This observation correlates with a previous study (Uzun et al., 2014). The diameters and cross-sectional areas of the seminiferous tubules appeared different, but the differences were not significant. There are significant differences in the lumen of the control and the group exposed to CAD, which shows CAD has the ability to disrupt the seminiferous tubules. There was significant difference in epithelium thickness of the CAD with or without NAP-treated groups. This shows that NAP does not have ameliorative effect on CAD-induced testicular damage.

CONCLUSION

Cadmium exposure led to reduction in the circulating level of Follicle stimulating hormone and Luteinizing hormone. Reduction in the serum level of this hormone will lead to inhibition of spermatogenesis and immaturity of spermatogonia, which can lead to male infertility. It can cause disruption of andrological parameters and the histo-architecture of the testicular histology. Furthermore, naproxen, an anti-inflammatory drug, is able to restore biochemical deficit, but does not restore testicular morphological alterations in cadmium-induced animals.

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