ORIGINAL ARTICLE

HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL CHARACTERIZATION OF ADULT TESTES EXPOSED TO DICLOFENAC SODIUM: THE ACUTE EXPOSURE SYNDROME

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ABSTRACT

Background:

Diclofenac sodium is a non-steroidal anti-inflammatory medication with prostaglandin synthesis inhibiting properties. It has long been used to treat a variety of testicular problems and symptoms. However, the use of NSAIDs for specific disorders has generated some concerns due to their ability to trigger apoptosis in a variety of cells.

Objective:

The goal of this study was to discover if Diclofenac sodium had any effect on the reproductive function of the testes.

Methods:

Ten male Wistar rats (160 – 200g weight) were randomly distributed into two groups designated as groups A and B respectively. The rats in group A were injected with physiological saline while the rats in group B were given 5mg/kg Diclofenac sodium (IM) for three days. Twenty-four hours (24hrs) after the administration of the last respective dose, the rats were euthanized using Ketamine (10mg/kg IM). The testes were excised, fixed in Bouin's fluid (48hrs), and subsequently processed according to the general techniques of tissue processing and microtomy as described by Bancroft and Gamble. Serial sections (5µm thickness) of the paraffin-embedded testicular tissue were obtained for the individual stains to be carried out.

Results:

Diclofenac sodium caused a considerable ($p<0.05$) loss in body weight of the experimental rats, as well as a significant ($p<0.05$) reduction in their sperm counts and motility, with an increase in the number of immotile sperm cells. When compared to the control group, rats given Diclofenac sodium showed a significant decrease in testicular carbohydrate, as well as decreased Ki67 and increased Cyclin D1 expression.

Conclusions:

Our data support the hypothesis that acute injection of Diclofenac sodium at a dose of 5 mg/kg for three days produces testicular injury and inhibits reproductive functioning. This paper discusses the immunohistochemistry effects of diclofenac sodium on testes.

Keywords: Diclofenac, Testes, Sperm, Ki67, Cyclin D1, NSAID

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INTRODUCTION

Certain medications and toxicants have been found to have negative effects on numerous organs and systems of the body, including the reproductive system, when used to treat specific health problems (1). Aspirin, acetaminophen, and prednisolone are examples of these medications. The extent of the harm caused by medication administration is determined by parameters such as exposure dosage and time (2). Renal failure, epithelial lining damage, liver damage, and kidney failure are among the health concerns linked to both chronic and acute exposures to these medicines (1,3).

Diclofenac is a non-steroidal anti-inflammatory medication (NSAID) with antipyretic, analgesic, and anti-inflammatory effects. It has been used to treat a variety of acute and chronic aches, as well as inflammatory disorders, in the past (4–6). However, due to their tendency to promote apoptosis in a variety of cells, the use of NSAIDs for some illnesses has raised some concerns (7). Vyas *et al* (2), for example, found aberrant histoarchitecture in male rats after 0.5mg/kg and 1.0mg/kg Diclofenac sodium treatment. Histopathological changes in the liver of diclofenac sodium-exposed mice have also been described (8). Increased exposure to toxicants and analgesics has been linked to the reproductive system and testicular organ damage (1,2), although the impact of this medicine on the male reproductive system of vertebrates like rats has not been well examined.

The use of diclofenac causes a significant dose-dependent reduction in testicular weights and deterioration in the functions of sperm parameters such as sperm count, motility and, density, as well as degeneration in the histoarchitectures of the testes, according to a previous study (9–11). Following a 30-day treatment with diclofenac sodium 1.0 mg/kg, Vyas *et al*. revealed significant reductions in sperm dynamics (count, motility, and density) in rats. Diclofenac sodium causes degenerations in germ cells and interstitial cells. Diclofenac sodium toxicity is demonstrated by aberrant histopathology of the testes and reduced testicular cell population dynamics.

The toxicological effects of numerous medicines, including diclofenac sodium, on the male reproductive system, have been suspected, and the mechanisms of interference have been examined using histological techniques. A study on the effects of diclofenac sodium (Voltaren) and prostaglandin E2 on spermatogenesis in mature dogs is one of them (12). The three steps of spermatogenesis are spermatogonial division and proliferation, spermatocyte meiosis, and spermatogenesis. Both PCNA and Ki67 antigen are found in the proliferating cell nucleus, and their expression levels can be used to assess spermatogenesis ability because they indicate the ability of spermatogonia and primary spermatocytes to proliferate, while their combination indicates the ability of spermatogonia and primary spermatocytes to proliferate (13). Ki67 is a non-histone nuclear protein that measures cell proliferation as well as the cell cycle. Cyclin D1 is also involved in the regulation of spermatogonia cell growth (14).

To our knowledge, there is still little or no literature on the effect of diclofenac sodium on the expression of proliferative markers (Ki67 and Cyclin D1) in the testes. As a result, further research into the impact of diclofenac sodium on the proliferative activities of Ki67 and Cyclin D1 in testicular tissues is required. Because of the potential harmful effects of diclofenac sodium on testes and a knowledge gap, the current study uses histochemical (PAS) and immunohistochemical staining techniques to evaluate the acute exposure syndrome of diclofenac sodium on male Wistar rats as it pertains to infertility.

METHODS

Experimental Animals, Feeding, and Care

All experiments were carried out according to the *Guide for the Care and Use of Laboratory Animals* by the National Institute of Health (NIH Publication, 2011). Ethical approval was sought from the Institution-based Ethical committee of the University of Ilorin, Ilorin, Nigeria. Ten adult healthy male Wistar rats (body weights between 160g and 200g) were purchased from the animal house of the University of Ilorin for the purpose of this study. The rats were kept in plastic

cages placed in a well-ventilated room under standard conditions with relative humidity and natural light and dark cycle. The rats were fed with a standard diet and clean water *ad libitum*. Throughout the study, the rats received humane care in accordance with the standard best practices.

Drug Preparation and Dose

Diclofenac sodium was purchased (from Aromokeye Pharmacy Ltd) in its liquid form (as 75mg/3ml) and diluted with normal saline as a vehicle. The daily therapeutic dose of 2mg/kg as required in an adult of about 70kg man was considered for the study (Yasmeen and Yasmin, 2016) while the acute dose was approximated as 5mg/kg Diclofenac sodium. Diclofenac sodium was administered intramuscularly for three (3) consecutive days.

Experimental Procedures

Animal Groupings and Sacrifice

Ten male Wistar rats (160 – 200g weight) were randomly distributed into two groups designated as groups A and B respectively. The rats in group A were injected with physiological saline while the rats in group B were given 5mg/kg Diclofenac sodium (IM) for three days. Twenty-four hours (24hrs) after the administration of the last respective dose, the rats were euthanized using Ketamine (10mg/kg IM). The testes were excised, fixed in Bouin's fluid (48hrs) and, subsequently processed according to the general techniques of tissue processing and microtomy as described by Bancroft and Gamble (15). Serial sections (5µm thickness) of the paraffin-embedded testicular tissue were obtained for the individual stains to be carried out.

Procedural determination of weight difference

Clinical signs of toxicity including changes in body weight were evaluated during administration as well as their feeding status. The initial body weights of the rats were taken using sensitive weighing balance at about twenty-four hours between the first administration while the final body weights were taken at about twenty-four hours after the last administration. The changes in body weights were calculated as "Final body weight - Initial body weight".

Estimation of Sperm count and motility

The testes were removed together with the epididymis and the latter was carefully separated. The cauda of the epididymis was severed from its remainder and quickly transferred to a pre-warmed slide (27°C) and lacerated with a razor. Sperms were characterized based on their morphology and motility. The progressive motility and morphology of the sperm cells were evaluated using the method outlined by (16). The sperm cells were counted with the use of the improved Neubauer chamber (Deep 1/10m; LABART, Munich Germany).

Procedure for sperm count and motility

Sperm motility was determined and characterized by counting the numbers of motile and immotile spermatozoa across the different selected fields that are not close to the edge of the cover-slip where motility may be altered (17).

Progressive motile sperm (approximate space gain of at least 5µm/s) was determined by counting only sperms that are in the field together at a particular moment with the exclusion of tailless heads and malformed sperms. They were further characterized into "rapid progressive motile" and "slow progressive motile sperms" using a difference in movement velocity of about 2.5µm/s existing between them (17). The remainders of the sperm cells within the same area were counted as non-progressively (<5µm/s space gain) motile and immotile spermatozoa (17).

The percentages of sperm motility were characterized and estimated by visualization using the low-power magnification (x10) of a phase-contrast microscope as outlined by Ademuyiwa *et al*(9). The results are expressed as percentages of progressive (rapid and slow), non-progressive and immotile spermatozoa (17).

Experimental procedure for determination of morphological grading

The morphology of spermatozoa was evaluated using eosin-nigrosine blue staining mixture (9). The spermatozoa were graded as normal sperm cells and abnormal sperm cells (with head defect, middle piece defect and, tail defect).

Haematoxylin and Eosin Staining

The protocol for Haematoxylin and Eosin staining technique as described by Bancroft & Gamble (15) was followed for this procedure. Tissue sections (5µm thickness) were cut from the paraffin blocks and stained with routine Haematoxylin and Eosin staining. Photomicrographs of the stained sections were taken and studied with a digital light microscope as explained by Ebokaiwe *et al* (16).

Histochemical Studies

Serial sections (5µm thickness) were obtained from the paraffin blocks and stained with Periodic Acid Schiff (PAS) for the histochemical evaluation of polysaccharides, neutral muco-substances and basement membranes.

Immunohistochemical studies

Formalin-fixed and paraffin-embedded testicular tissues sectioned at 4µm were stained using Avidin Biotin complex (ABC) method for the immunohistochemical localization of Ki67 and cyclin D1 according to Sakr & Nooh (18). Areas of positive expression of Ki67 and Cyclin D1 were identified through intense brown staining (19).

ImmunoRatio web application was used to analyze nuclear immunostains in the seminiferous tubules as reported by (20). The immunoratio software application utilizes the colour deconvolution algorithm that differentiates the staining component (DAB chromogen and Haematoxylin counterstaining) and adaptive thresh holding for nuclear area segmentation to compute the percentages of positively stained nuclear area (16). The ImmunoRatio software (version 1.0c) was used to quantify the positive slides of ki67 and cyclin D1 (21). The immune-interaction sites were reflected with immune tinctures which are indicative of the occurrence of cell proliferation (21).

Statistical Analysis

Statistical analysis was performed using Graphpad Prism 5.0.1 software. The sperm counts were plotted using the unpaired t-test. Other data for sperm characteristics were recorded in tabular form. Statistical significance of $p<0.05^{*}$ (95% C.I) was used. Results were represented in bar charts with error bars to show the mean and standard error of mean respectively.

RESULTS

Changes in body weight and sperm parameters

When compared to their respective control group, the diclofenac-treated group demonstrated a significant drop in body weight ($p<0.05$) (Fig 1). In addition, diclofenac sodium delivery resulted in a substantial ($p<0.05$) drop in sperm counts, as well as rapid and slow progressive motilities respectively (Figs. 2, 3, 4). When diclofenac-administered rats were compared to the control group, the number of sperm with non-progressive motility (Fig. 5; Table 1) and immotile sperm cells (Fig. 6; Table 1) increased significantly ($p<0.05$). The diclofenac-treated group displayed a significant reduction ($p<0.05$) in the number of normal sperm cells in comparison to their respective control group (Fig 7; Table 2) and a corresponding significant increase ($p<0.05$) in the number of sperm cells with head defects, mid-piece defects and tail defects (Fig. 8, 9 and 10; Table 2) respectively when compared with control.

Histopathological Results

The morphology of the seminiferous tubules (Fig. 12a) revealed well-organized germinal layers of spermatogenic cells, Sertoli cells, normal lumen containing mature spermatozoa, and Leydig cells (of the interstitium), whereas the seminiferous tubules of rats given diclofenac sodium showed significant damage in the rat's testes, characterized by marked dilatation and presence of more vacuolated areas (Fig. 12b). There was a significant reduction in mean Johnson scores ($p<0.05$) when comparing the seminiferous tubules of rats given diclofenac sodium to those of the control group (Table 3; Fig. 11).

The presence of mucopolysaccharides in the rats' testes was revealed by a PAS-stained response. The testes of control rats (Fig. 13a) displayed a robust PAS-positive reaction that was localized in the spermatogenic cells, basement membrane, and Leydig cells, in contrast to the diclofenac-treated rats' mucopolysaccharide content being significantly reduced (Fig. 13b).

In the control group, the basal germ cells (spermatogonia) and Leydig cells of the testes of rats showed positive immunostaining (brown nuclear reaction) (Fig. 14a). When comparing the area of DAB expression of Ki67 stained testicular tissue of rats given diclofenac sodium to that of the control group, immunoratio analysis revealed that the area of DAB expression of Ki67 stained testicular tissue of rats given diclofenac sodium was reduced (Table 4; Fig. 14b).

In the control group, Cyclin D1 was expressed positively in the spermatogonia, basement membrane, and a little quantity within the lumen of the seminiferous tubule of the testes of rats (Fig. 15a). When compared to the control group, the expression of Cyclin D1 increased in the diclofenac sodium group. As spermatogenic cells assemble towards the lumen, Cyclin D1 was expressed in the basement membrane and the spermatogenic cells (Fig 15b). When comparing the testicular tissue of rats given diclofenac sodium to that of the control group, immunoratio analysis revealed a significant increase in the area of DAB expression in the Cyclin D1-stained testicular tissue of the diclofenac sodium-treated rats (Table 5).

DISCUSSION

Because of their analgesic and anti-inflammatory properties, non-steroidal anti-inflammatory medications (NSAIDs) are commonly utilized in the treatment of various testicular illnesses. Diclofenac sodium is one of the most often prescribed medications, with its prescriptions steadily increasing from 2006 (126th) to 2016 (78th) (22,23). This is one of the few research we are aware of that has looked into the reproductive effects of diclofenac sodium on the testes. The researchers anticipated that acute diclofenac sodium injection has some negative impacts on the testes' reproductive function. The current study looked at how diclofenac-induced toxicity affected body weight, sperm parameters, morphological, histochemical, and immunohistochemical changes in adult rats' testes.

According to our data, body weight alterations in rats following diclofenac sodium injection showed a significant decrease (Fig. 1). The weight loss can be attributed to "diclofenac sodium hyper-accumulation with direct cytotoxicity effect on testes tissues" or "seen decreased food intake, nutrients malabsorption from the colon, and impaired feeding conversion." This could be linked to the fact that diclofenac sodium has a considerable impact on the hypothalamic endocrine function, which regulates eating and water intake. This is consistent with the findings of Ademuyiwa *et al* on experimental animals exposed to environmental toxins, as well as Yousef *et al* who found that Wistar rats' body weight was reduced after exposure to cypermethrin.

As a marker, semen quality and testicular function can be used to determine general health (24). Individuals with a low sperm count of less than 39 million per ejaculate are at a higher risk of hypogonadism and are more frequently connected with lipid and glucose dysmetabolism, according to research (24). The sperm counts of diclofenac-treated rats were found to be significantly lower (Fig. 2). Also in the testes of diclofenac-treated rats, we found a significant decrease in the quantity of sperm with progressive motility (Table 1; Fig. 3&4) and a comparable rise in the number of non-progressive and immotile sperm cells (Table 1; Fig 5&6). These changes in sperm motility have the ability to wreak havoc on reproductive functions and even lead to fertilization failure. Progressive motility is one of the most significant characteristics of human sperm ejaculate because it dictates their capacity to penetrate and migrate through the cervical mucus and the cellular components that surround the oocyte to allow fertilization to occur (17).

Anti-inflammatory medicines have been shown to suppress prostaglandin synthesis, which may lead to sperm motility alterations (25,26). As a result, the increase in the number of immobile sperm cells and sperm counts in diclofenac-treated rats can be linked to changes in prostaglandin production and testosterone levels. Our findings were consistent with those of Mousa *et al* (27) who found a substantial decrease in reproductive sperm parameters such as sperm count and motility after an 8-week oral dosage of 2.5 mg/kg diclofenac sodium (4 times per week). We also found a significant decrease in the quantity of sperm with normal

morphology (Table 2; Fig. 7) and a corresponding significant increase in the number of sperm cells with abnormalities (head, middle piece, and tail) (Table 2; Fig. 8-10). The examination of sperm structural abnormalities is particularly significant during the evaluation of male fertility potential because abnormalities in sperm morphology might be considered indications of male fertility (28). Alterations and impairment of the sperm nucleus DNA and proteins may have caused changes in sperm morphology. Sperm proteomics revealed the importance of sperm shape and the many proteins found in each location. Certain proteins, such as ENO1, CRISP2, SHTAP, VDAC2, VDAC3, and others, have been found in the tail portion of sperm cells, whereas others, such as GAPDH, PKA, AVPR2, and ropporin, have been found in the mid-piece (29). As a result, interference with the metabolic activities of any of these proteins by diclofenac sodium is likely to cause "immotility when proteins in the tail-piece are involved" or "energy chain breakdown when proteins in the mid-piece are involved."

The current investigation of the histopathological results of diclofenac-injected rats (Fig. 12) revealed alterations in the seminiferous tubules, including destruction and depletion of spermatogenic cells, Sertoli cells, vacoulation, and degeneration of the Leydig cells in the testes, which are consistent with previous research findings (27,30). There was a considerable drop in spermatogenic cells when Johnson's scoring system was used on the seminiferous tubule (Table 3; Fig 11). This study implies that diclofenac sodium inhibits spermatogenesis, which could be linked to a decrease in testosterone levels and spermatogenic process-regulating activities. Normally, Leydig cells produce testosterone to manage the testes' reproductive activity (31). Because of the near closeness of the Leydig cells to the blood arteries, they are at a high risk of circulating medicines (30). Prostaglandin is known to play an essential role in the maturation of sperm cells (26), and LH, which works on the Leydig cells, stimulates the creation of its precursor (arachidonic acid) and testosterone (30). As a result, diclofenac sodium, a nonsteroidal anti-inflammatory medicine that has been shown to interfere with COX-2 (26), will impact the activity of Leydig cells, resulting in lower testosterone levels. The morphological changes in the Sertoli cells' structure, as well as the sloughing of the germ cells and the dilated intercellular space, indicate that the Sertoli cells' function and structure are defective (30).

Sertoli cells are recognized for their role in forming the blood-testis barrier, which protects germ cells from cellular insults. They are also in charge of supplying nutrients and hormones to germ cells (32). Changes in the Sertoli cell microenvironment are reflected in the testes of diclofenac-treated rats, which include degeneration and vacoulations in the seminiferous tubules, which in turn disrupts the mechanism of protein production essential for germ cell development (30). Our findings are similar to those of Obeys *et al* (30) and Mousa *et al* (27), who found degenerative alterations after acute diclofenac sodium treatment. According to Campion *et al*, Sertoli cell disruption may have caused testicular tissue disturbance. Treatments with 0.5mg/kg and 1.0mg/kg diclofenac in mice for thirty days resulted in vacoulation, degeneration, and apoptosis (spermatids, primary & spermatocytes, spermatogonia, Sertoli cells, and Leydig cells) in the seminiferous tubules (11). These modifications may cause androgen activity to be disrupted as a result of cellular damage (7,30,33).

In the current investigation, the PAS-positive reaction in the basement membrane of the seminiferous tubules and interstitial cells was found to be significantly reduced. The Periodic Acid Schiff staining technique is a histochemical reaction that is used to show the carbohydrates content of tissues (such as testes) (34). Testicular glycogen depletion can be due to suppression of phosphorylase activation or changes in other enzymes that impede androgen production. A decrease in PAS-positive particles in sperm cells may indicate a metabolic problem in the testes. Because the glucose transporter is the principal mechanism of glucose uptake in the seminiferous tubules, any degenerative event there is likely to result in changes in cellular glucose metabolism. Spermatozoa motility is reliant on a steady supply of energy and a jealously guarded polyunsaturated fatty acid membrane that is

vulnerable to oxidative stress damage. As a result, the rats' reduced food intake may have resulted in a loss of energy and, as a result, a breakdown of stored body glucose. Furthermore, diclofenac-induced aberrant sperm morphology, such as a middle-piece deficiency that results in slow motile or immotile sperms, could be connected to insufficient glucose supply and metabolism to the mitochondria, which serve as the cells' powerhouse. Reduced carbohydrates intake causes a metabolic shift from glucose to lipids (35), a process known as gluconeogenesis, which can affect the creation of important proteins like Ki67 and cyclin D1. For optimal testicular activity and, in general, for energy production, a constant supply of glucose is required. The decrease in carbohydrate content correlates to a decrease in energy production, which might be linked to weight loss in the body and organs. According to Yousef *et al* (36), the buildup of blood glucose after pesticide treatment indicates a change in carbohydrate metabolism as a result of compensation from liver glycogen degradation. Due to a lack of carbohydrates, this could have been mediated by the over-expression of glucagon hormones and adrenocorticotrophic hormones. It has also been reported that Ciprofloxacin has the ability to change the biochemical integrity of exposed cells even at low doses, according to Zobeiri et al (35).

Proliferative markers were used in this study to learn more about diclofenac sodium's suppressive effects on testicular tissues. According to our findings, there was a reduction in the area of DAB expression in Ki67-stained testicular tissue of rats given diclofenac sodium (Table 4; Fig. 14b). Steger *et al* found a link between decreased Ki67 protein expression and spermatogonia proliferative activity as well as germ cell loss during mitosis and meiosis. Because they are expressed throughout late G1-, S-, G2-, and M-phases of the cell cycle and absent in resting cells and early G1-phase (Miller *et al*., 2018), Ki-67 has been utilized as a biomarker for estimating the rate of proliferative cells (37,38). Ki67 has been linked to major nuclear structural changes as cells enter and exit mitosis (38). As a result, the detrimental effect of acute diclofenac sodium injection can be linked to a considerable drop in Ki67 expression in the seminiferous tubules and interstitium. This shows that Diclofenac sodium inhibits Ki67's proliferative activity in testicular tissue, resulting in a decrease in DNA synthesis in pathologic testes and, as a result, germ cell loss during meiosis.

We also discovered that Cyclin D1 was more positively expressed in the diclofenac-treated group. Cyclin D1 expression in the testes corresponds with spermatogonia and gonocyte G1/S progression, which acts as a checkpoint (39). According to Pagano *et al*., over-expression of cyclin D1 hinders DNA repair. Cyclin D1 expression in spermatogonia across stages of the seminiferous epithelium cycle implies that they are important regulators of proliferating spermatogonia and gonocytes, particularly during the G1/S phase transition. The expression of cyclin D1 has been found to increase under two cell cycle conditions namely cell growth and cell cycle arrest (39). In the absence of Cdk4 or Cdk2, Pagano *et al* found that co-expression of cyclin D1 and PCNA leads to over-expression of Cyclin D1, which inhibits DNA repair by around 50%. Cyclin D1 is a key regulator of cellular proliferation that connects the extracellular signaling environment to cell cycle progression (40). The action of proliferative signals such as growth factor receptors, Ras, and their downstream effectors has a big impact on Cyclin D1 expression (40). As monitoring proteins, they play an important role in cell proliferation and cell arrest/apoptosis (41). In mammals, three distinct cyclins (D1, D2, and D3) have been found. The increase of cyclin D1 protein, as a result, suggests diclofenac sodium's capacity to activate and stimulate apoptotic pathways.

CONCLUSION

Overall, this research was focused on examining the effects of acute administration of diclofenac sodium. Diclofenac sodium injection resulted in a significant reduction in body weight in rats. The sperm counts of diclofenac-treated rats were found to be significantly lower. The amount of sperm with progressive motility decreased significantly, while the number of non-progressive and immotile sperm cells increased. The number of sperm cells with abnormalities grew dramatically, while the number of sperm with normal

morphology declined significantly. Seminiferous tubules and Leydig cells in diclofenac-treated rats showed degenerative changes. Histochemical findings revealed that diclofenac-treated rats had a considerable reduction in PAS-positive particles in their sperm cells. Immunohistochemical examination further revealed a decrease in Ki67 expression with a matching increase in Cyclin D1 expression. The results of the preceding study demonstrate that giving Diclofenac sodium at a level of 5 mg/kg for three days induces testicular injury and inhibits reproductive capabilities. As a result, this study adds to the body of knowledge on the immunohistochemistry examinations of diclofenac sodium's acute effects on adult Wistar rats' testes, as well as to the body of knowledge on the drug's harmful effect on the reproductive system. Future research should concentrate on the possibility of employing conjugate medicines to alleviate these side effects.

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Table 1: Sperm Motility grading (in percentages)

PARAMETERS	Group A (control)	Group B (3 days Diclofenac Sodium)
Rapid progressive motility	37.60 ± 1.600	25.60 ± 2.040
p-value	<0.0001	0.0002
Slow progressive motility	39.20 ± 1.497	25.60 ± 2.040
p-value	<0.0001	0.0002
Non-progressive motility	13.60 ± 0.9798	22.40 ± 0.9798
p-value	0.0002	<0.0001
Immotile sperm cells	9.600 ± 0.9798	26.40 ± 1.600
p-value	0.0006	<0.0001

Mean value ± SEM (standard error of mean). The mean difference is significant at P<0.05

Table 2: Sperm Morphology grading (in percentages)

PARAMETERS	Group A (control)	Group B (3 days Diclofenac Sodium)
Normal Sperm Cell	92.00 ± 1.265	75.20 ± 1.497
p-value	<0.0001	<0.0001
Head defect	2.200 ± 0.4899	7.600 ± 0.7483
p-value	0.0109	0.0005
Mid-piece defect	1.800 ± 0.2000	6.400 ± 0.7483
p-value	0.0008	0.0010
Tail defect	4.000 ± 0.6325	10.80 ± 0.4899
p-value	0.0032	<0.0001

Mean value ± SEM (standard error of mean). The mean difference is significant at P<0.05

Table 3: MEAN JOHNSON SCORES (%)

	Group 1	Group 2
Mean ± SEM	9.600 ± 0.2449	6.400 ±0.2449
p-value	<0.0001	<0.0001

Mean value ± SEM (standard error of mean). The mean difference is significant at P<0.05

Table 4: Statistical Summary table for Immunoratio of Ki67

	Group 1	Group 2
DAB/nuclear area	9.6%	8.3%

Table 5: Statistical Summary table for Immunoratio of Cyclin D1

	Group 1	Group 2
DAB/nuclear area	14.9%	63.8%

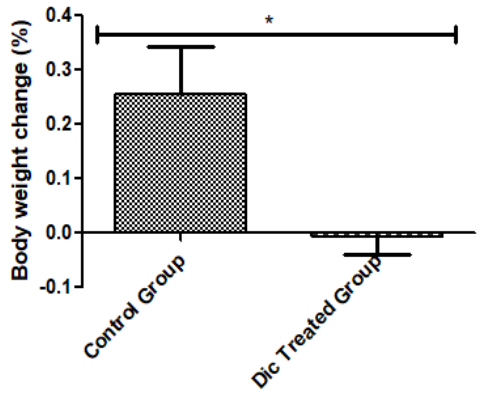

Figure 1: Effects of Diclofenac sodium on body weight changes in control rats (A) and exposed rats (B). Each bar represents mean ± SEM of five rats. *p<0.05 compared to control.

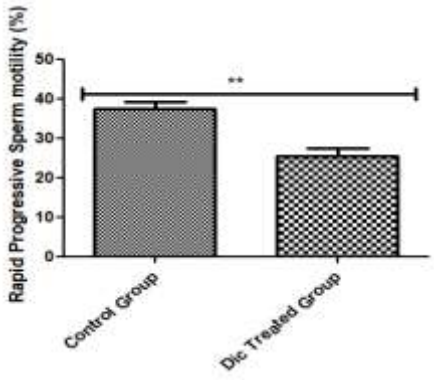

Figure 3: Effects of Diclofenac sodium showing differences of Rapid Progressive Sperm motility in control rats (A) and exposed rats (B). Each bar represents mean ± SEM of five rats. *p<0.05 compared to control

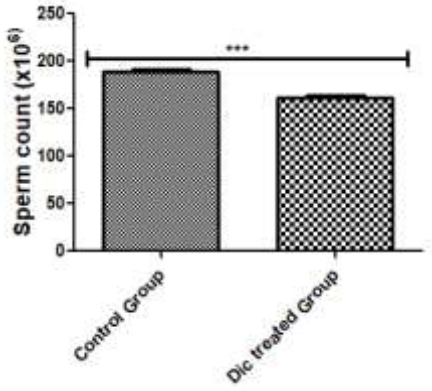

Figure 2: Effects of Diclofenac sodium on Sperm counts in control rats (A) and exposed rats (B). Each bar represents mean ± SEM of five rats. *p<0.05 compared to control

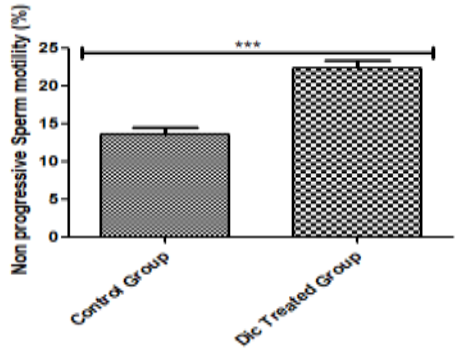

Figure 5: Effects of Diclofenac sodium showing differences of Non-progressive sperm motility in control rats (A) and exposed rats (B). Each bar represents mean ± SEM of five rats. *p<0.05 compared to control

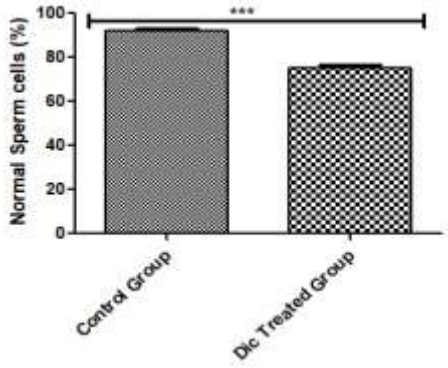

Figure 7: Effects of Diclofenac sodium showing differences in normal sperm cell morphology in control rats (A) and exposed rats (B). Each bar represents mean ± SEM of five rats. *p<0.05 compared to control

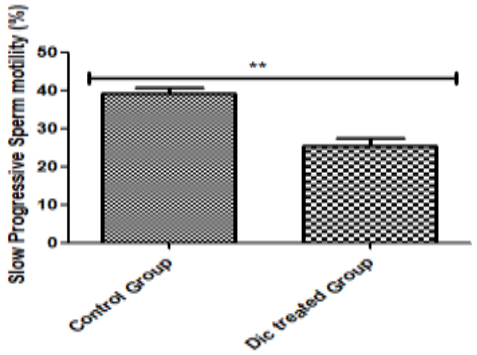

Figure 4: Effects of Diclofenac sodium showing differences of slow progressive sperm motility in control rats (A) and exposed rats (B). Each bar represents mean ± SEM of five rats. *p<0.05 compared to control

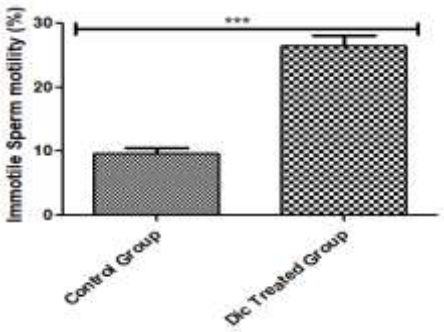

Figure 6: Effects of Diclofenac sodium showing differences of Immotile Sperm cell morphology in control rats (A) and exposed rats (B). Each bar represents mean ± SEM of five rats. *p<0.05 compared to control

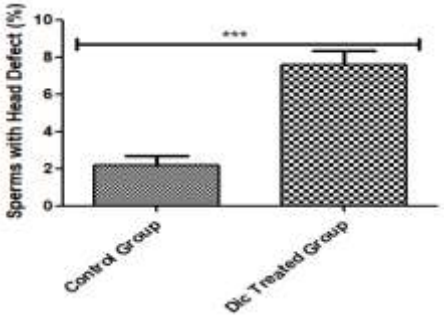

Figure 8: Effects of Diclofenac sodium showing differences in sperm cell with Head defect in control rats (A) and exposed rats (B). Each bar represents mean ± SEM of five rats. *p<0.05 compared to control

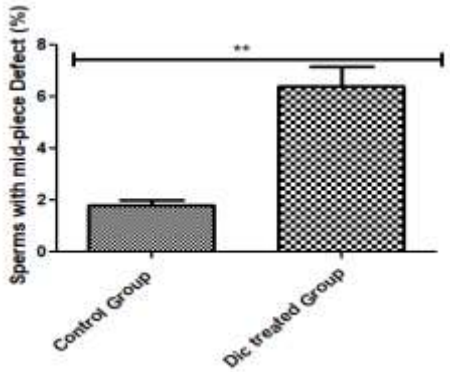

Figure 9: Effects of Diclofenac sodium showing differences in sperm cell with Mid-piece defect in control rats (A) and exposed rats (B). Each bar represents mean ± SEM of five rats. *p<0.05 compared to control

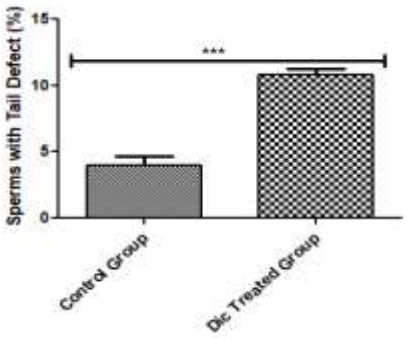

Figure 10: Effects of Diclofenac sodium showing differences in sperm cell with tail defect in control rats (A) and exposed rats (B). Each bar represents mean ± SEM of five rats. *p<0.05 compared to control.

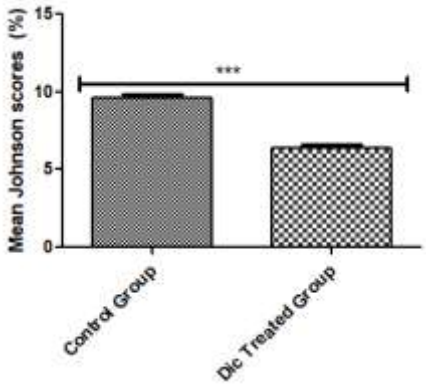

Figure 11: Effects of Diclofenac sodium showing differences in Mean Johnson's score in control rats (A) and exposed rats (B). Each bar represents mean ± SEM of five rats. *p<0.05 compared to control

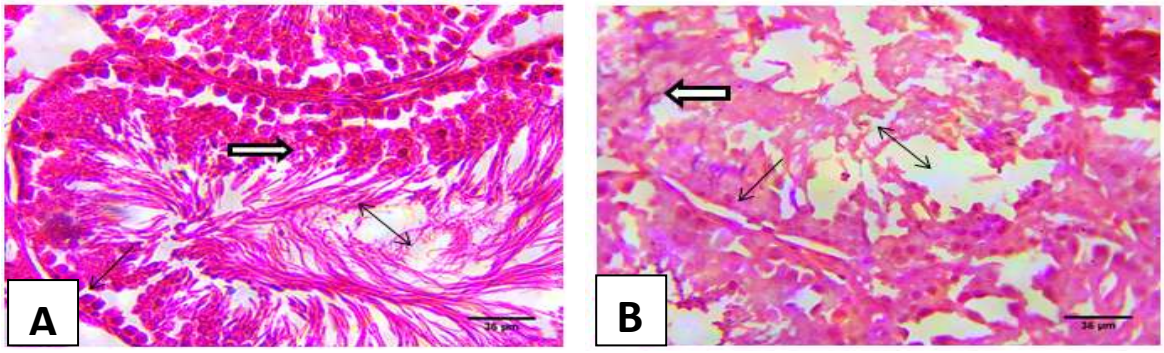

Figure 12: General morphology of the germinal epithelium of adult wistar rats stained with H&E (x800). a) Control, White arrow (⇨) showing normal spermatocytes; arrow (→) showing normal spermatogonia cells on the basement membrane; double arrow (↔) indicates normal mature sperm cells in the lumen. b) 10mg/kg Diclofenac sodium (3 days), White arrow (⇨) indicates distortion of the spermatogonic cells and majorly the spermatocytes; arrow (→) indicates distortion and depletion of spermatogenic cells on the basement membrane; double arrow (↔) indicates gaps seen in the arrangement of spermatogenic cells which shows huge loss of spermatogenic activities (as a result of damage to Sertoli cells).

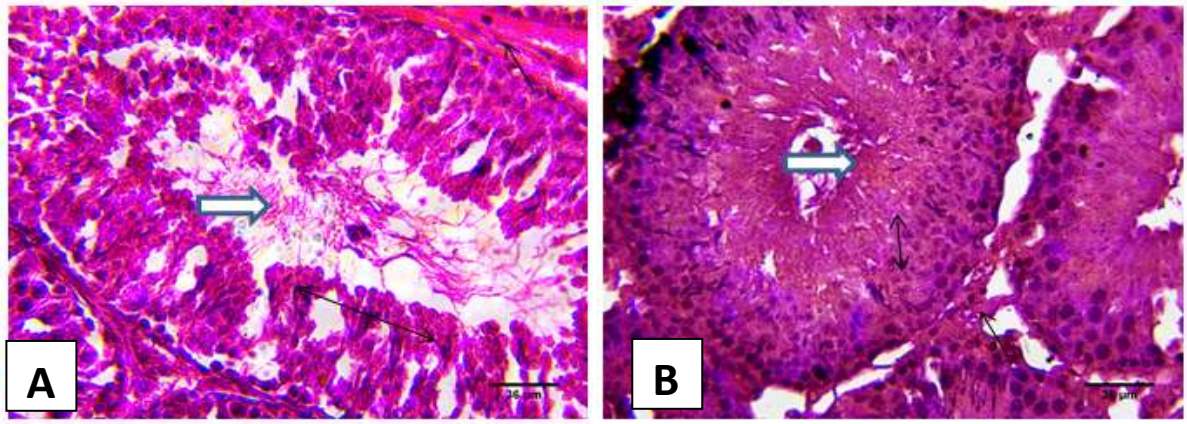

Figure 13 (a-b): Effects of Diclofenac sodium on morphology of the germinal epithelium of adult wistar rats (stained with PAS x800) in control rats (A) and exposed rats (B). Each bar represents mean ± SEM of five rats. *p<0.05 compared to control.

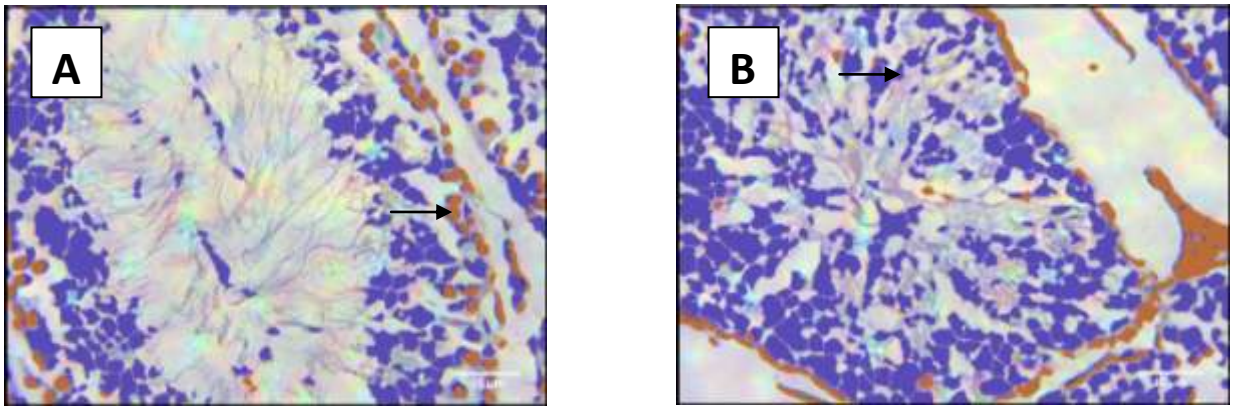

Figure 14: Immunoratio analysis of Ki67 immunohistochemical staining with DAB as the chromogen. Testicular expression of Ki67 in the germinal epithelium of adult wistar rats. A) ImageJ analysis showing area of DAB expression in seminiferous tubule of the control group. Ki67 expression was distinctly observed among the spermatogonia cells (⟶). B) ImageJ analysis showing area of DAB expression in the basement membrane, spermatogonia, spermatocytes and interstitial cells of animals administered with diclofenac sodium. There was variation (reduction) in Ki67 expression across the spermatogenic cells.

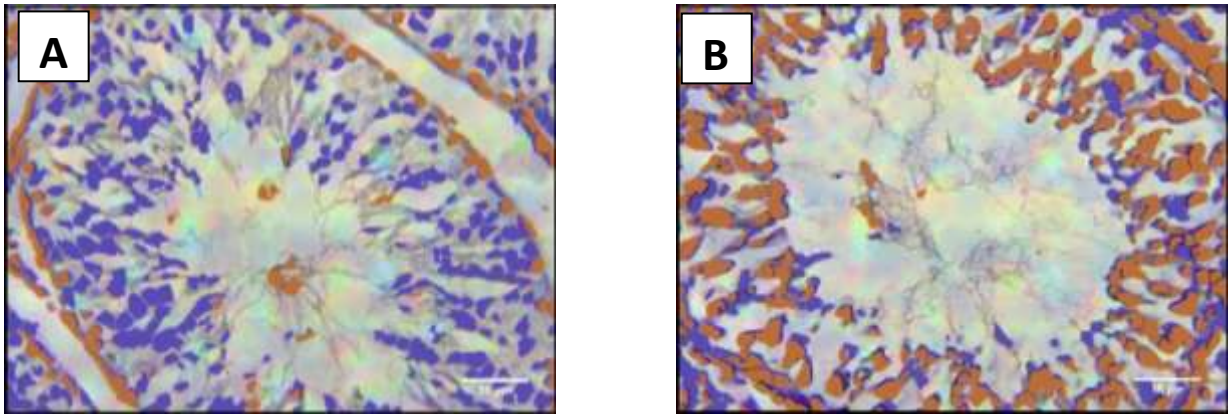

Figure 15: Immunoratio analysis of Cyclin D1 immunohistochemical staining with DAB as the chromogen. Testicular expression of Cyclin D1 in the germinal epithelium of adult wistar rats. A). ImageJ analysis showing area of DAB expression in the control group where Cyclin D1 expression was greatly observed in the spermatogonia and basement membrane with less expression in the lumen. B) ImageJ analysis showing area of DAB expression in the group administered with diclofenac sodium where Cyclin D1 was greatly observed across the basement membrane, spermatogonia, spermatocytes and interstitial cells.