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Immunohistochemical and Stereological Studies of *Treculia africana* Seed on Chloroquine Induced Damage on the Testes of Adult Male Wistar Rats (*Rattus novergicus*)

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Abstract: *Introduction*: Chloroquine causes damage to the testis by crossing the blood testis barrier. *Treculia africana* has numerous medicinal potentials. However, the prevention of its complication such as male infertility remains unexplored. This aim of this study was to evaluate the effects of aqueous seed extract of *Treculia africana* on chloroquine-induced toxicity in the testis of adult male Wistar rats (*Rattus norvegicus*). *Methods*: Twenty eight (28) rats with an average weight 200±10g were used for the study and divided into four groups consisting of seven rats in each group. Control group A received 1 ml of distilled water daily, group B received 2mg/kg body weight daily of chloroquine orally, group C received 200mg/kg body weight daily of aqueous seed extract of *Treculia Africana* while group D received 2mg/kg body weight daily of chloroquine and *Treculia africana* seed extract (TASE) concurrently for a period of 60 days. Histological, histopathological, hormonal and semen analysis, Stereological and Imunohistochemical evaluation of the testis were done. *Results*: Histological analysis showed the ameliorative properties of TASE after chloroquine induced testicular toxicity. Significant differences ($P<0.05$) in hormonal analysis were observed across treatment groups. Significant differences ($P<0.05$) in Semen and Sterological analysis were observed. Johnsen's Score of the testis showed a significant improvement in group D while Immunohistochemical observation showed the ameliorative properties of TASE. *Conclusion*: chloroquine is injurious to male reproductive health, administration of *TASE* may help to improve seminiferous tubule integrity and immunohistochemistry and stereological studies further revealed its ameliorative properties.

Keywords: *Treculia africana* Seed Extract (TASE), Chloroquine, Testis, Immunohistochemical, Stereology

1. Introduction

Chloroquine belongs to the quinolone family with different therapeutic and toxic doses [1]. It was initially given as a prophylaxis and treatment for malaria and later used to treat different ailments. Chloroquine toxicity has led to the formation and production of reactive oxygen species Mohktar et al. [2] while its adverse effects like cardiotoxicity, allergies, skin and hair lesions, oculotoxicity, hepatorenal toxicity and disorders of the gastrointestinal tract have been recorded [3]. Currently, few studies have reported the toxic effects of chloroquine on male reproductive function and morphology [4]. Elgndy et al. [5] reported that chloroquine induction had orchidotoxic effects on male reproductive functions. On the other hand there are limited data regarding the adverse effects of chloroquine on male reproductive system [6]. Several interventions have been carried out in the prevention and management of testicular damage and one of the integral components is tradomedicine.

African breadfruit known as *Treculia africana* is a tropical African plant with nutritional and ethnomedicinal valence [7]. Different reports on phytochemical screening of TASE showed that it constitutes anthraquinones, cardiac glycosides, polyphenols, saponins and flavonoid [8]. In the Middle and

Western Belts of Nigeria, the plant has been reported to be an important component for the treatment of diabetes [9].

Thus, this study investigated the potential ameliorative effects of aqueous seed extract of *Treculia africana* on chloroquine-induced toxicity on the testis of adult male Wistar rats (*Rattus norvegicus*).

Ethical Approval

Ethical approval was obtained from the Research and Ethics Committee of the Faculty of Basic Medical Sciences, University of Ilorin, Ilorin, Nigeria.

2. Materials and Methods

2.1. Preparation of Seed Extract

Mature fruit samples of *Treculia africana* were collected from a rainforest in Imo State, Nigeria and properly, identified at the University of Ilorin Botanical Garden. Seeds were manually separated from five freshly plucked fruits and oven dried in an Astell Hearson type oven at 61°C for 24hr (figure 1). The dried samples were pulverized with an electric grinder into powdered form. 200 g of the powdery material was stored under room temperature before use and prepared in 100 ml of distilled water. It was then filtered through whatman No. 1 filter paper and the excess water removed using a rotary evaporator and stored in the refrigerator for use. The ethanolic extract was obtained by Soxhlet extractor followed by evaporation and concentration to remove the alcohol [8].

Figure 1. *Showing freshly plucked breadfruits.*

2.2. Animal Grouping and Drug Administration

Twenty-eight (28) adult male Wistar rats, weighing 200g were purchased and maintained in the animal house of the College of Health Sciences, University of Ilorin. An acclimatization period of two weeks was carried out while the rats' weight was carried out on a weekly basis using an Electronic Weight Balance in the Department of Anatomy, University of Ilorin. The rats were divided into four groups which consists of seven (7) rats in each group as follows:

1) Control group A received 1 ml of distilled water daily.
2) Group B received 2mg/kg body weight daily of chloroquine orally.
3) Group C received 200mg/kg body weight daily of aqueous seed extract of *Treculia africana* seed extract (TASE).
4) Group D received 2mg/kg body weight daily of chloroquine + 200mg/kg body weight daily of *Treculia africana* seed extract (TASE) orally for 60 days.

2.3. Animal Sacrifice and Sample Collection

24 hours after day 60, the animals were sacrificed; blood samples were collected through cardiac puncture and placed into lithium heparinized bottles. Blood serum for hormonal analysis were collected and centrifuged at 3000 rpm for 15 mins.

2.4. Histological Analysis

The right testes was fixed in Bouin's fluid and processed for light microscopic examination. Histological staining was carried out using Haematoxylin and Eosin methods as described (Bancroft and Stevens, 1996). Slides were taken with the aid of an AmScope Digital Microscope (AmScope Microsystem, USA) connected to a computer [10].

2.5. Semen Analysis

Sperm concentration [11], motility [12], morphology plus life and death ratio [13] were analyzed using the Neubauer Chamber Haemocytometer (Deep 1/10 mm, LABART, Germany).

2.6. Biochemical Assay

Serum follicle stimulating hormone (FSH), testosterone and Luteinizing hormone (LH) concentration were determined using Accu Bind ELISA Microwell by Monobind Inc. Lake Forest, CA 92630, USA.

2.7. Procedure for Immunohistochemistry

Proliferation of spermatogenic cells was tested using the Anti-Ki67 antibody (SP_6) ab16667. Excised testes were fixed with Bouin's fluid and a mediated heat antigen retrieval step was done using citrate buffer. Samples were blocked with 1% bovine serum albumin for 10 mins at 21°C followed by incubation with the primary antibody for 2 hours at 1/100. A goat biotin conjugated anti-rabbit polyclonal agent was used as secondary antibody at a 1/250 dilution.

2.8. Histopathological Analysis (Johnsen's Score) of the Testis

Histological sections stained with H and E was employed for histopathological analysis of testicular tissue to determine effect of treatment and exposure to chloroquine and TASE. In addition to qualitative descriptions, the testicular histology was analyzed quantitatively using modified Johnsen's Score. It grades the seminiferous tubules on a scale of 1-10, so that the best histological appearance with presence of full spermatogenesis is scored 10 while the worst histological appearance with the absence of seminiferous tubule is given a score of 1 [14]. The modified Johnsen scores were applied to a minimum of 25 randomly chosen seminiferous tubules from each animal. The mean score for each animal was

obtained by multiplying the numbers of tubules awarded a score with the score and, then, the total number of tubules graded was divided by the sum of all the multiplications. The group mean was obtained by dividing the sum of means by number of animals per group.

2.9. Histomorphometric (Stereological) Evaluation of the Testis

Testicular tissues were sectioned at 3 µm and stained with H and E for stereological studies. Histomorphometric data were collected with the aid of an Amscope digital microscope (Amscope Microsystem, USA) connected to a computer [10]. Evaluation of seminiferous tubule volume density, epithelium height, seminiferous tubule diameter, cross-sectional area of the seminiferous tubules, numbers of seminiferous tubules profiles/unit area of testis and numerical and length densities of the seminiferous tubules. Each sampled section was analyzed using a video microscopy system made up of a microscope (E-200, Nikon, Japan) linked to a video camera (Sony, Japan, SSC Dc 18P), a computer, and a flat monitor. On average, for estimating each parameter, ten to fourteen microscopic fields were examined in each testis for any estimation. In selection of microscopic fields, a systematic random sampling procedure was applied (i.e., the position of the first field was selected randomly outside the sections) on one corner of the microscopic slide. Then, the slide was moved at equal intervals along the x- and y-axis using a stage micrometer. This procedure was continued until all of the sections had been studied. Test probe or stereology grids were laid on the microscopic images by means of the stereology software.

2.9.1. Seminiferous Tubule Estimation and Germinal Epithelium Volume

To estimate the germinal epithelium and seminiferous tubules total volume, 5-m sections were used. A multicascade design was applied to estimate the stereological parameters. A grid of points was overlaid on the monitor image of the testis. At the first level of multicascade estimation, the volume density of the tubules ($Vv_{(tubule/testis)}$) or the ratio of the testis volume occupied by tubules was obtained using point counting at final magnification of 19 X and the following formula:

$$Vv_{(tubule/testis)} = P_{(tubules)} / P_{(testis)}$$

where the P (tubules) and P (testis) were the number of points hitting the profiles of the tubules and testis, respectively. It is only in the knowledge of the size (volume) of the reference space that the character of any variation (or lack of variation) can be fully understood according to methods [15].

2.9.2. Statistical Analysis

Data collected were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's (HSD) multiple comparison test with the aid of SPSS (V20; USA) [10]. P value ($p<0.05$) was considered significant statistically. Graphs were drawn using the GraphPad Prism v. 6 (GraphPad Software Inc., USA).

3. Results

3.1. Body Weight Changes

No significant difference ($P>0.05$) in the final body weight across all the treatment groups when compared with the control group (figure 2) in this study.

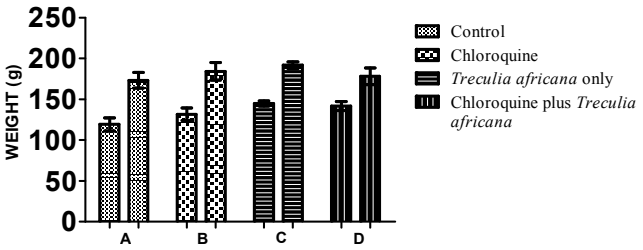

Figure 2. *Showing the initial and final weight changes in the rats.*

3.2. Semen Analysis

Significant decrease ($P<0.05$) in concentration of sperm (figure 3), morphology, motility, and Life/Death ratio were observed in group B alone while no significant differences ($P>0.05$) in groups C and D was observed compared with the control group (figure 4).

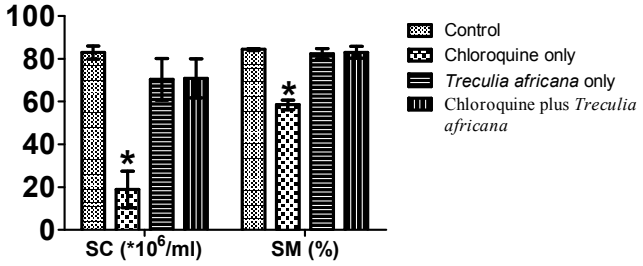

Figure 3. *Showing Sperm concentration and Sperm motility in the treated and control groups. *(P<0.05)- statistical significant.*

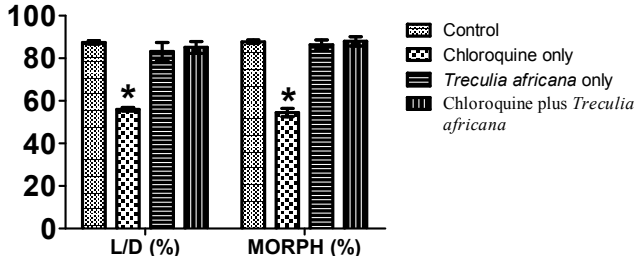

Figure 4. *Showing life/death ratio and sperm morphology in treated and control groups. *(P<0.05)- statistical significant.*

3.3. Serum Hormonal Levels

Significant increase ($P<0.05$) in follicle stimulating hormone levels (FSH) was observed in group C when compared with the control group (figure 5) while in group D a significant decrease ($P<0.05$) in luteinizing hormone (LH) levels was observed when compared with the control (figure 5). No significant differences ($P>0.05$) in testosterone levels across all the treatment groups were observed compared with

the control group (figure 6).

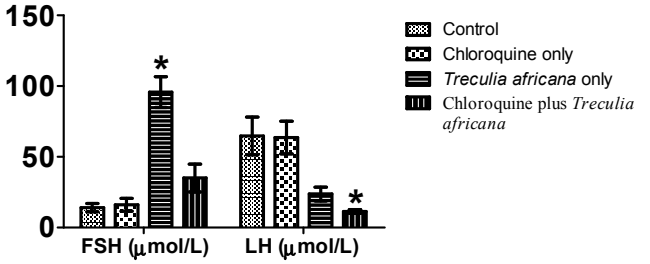

Figure 5. *Showing the follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels in treatment and control groups. *(P<0.05)- statistical significant.*

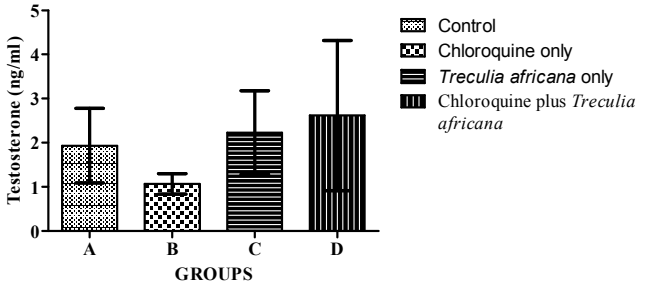

Figure 6. *Showing Testosterone levels across all groups. *(P<0.05)- statistical significant.*

3.4. Stereological Analysis

3.4.1. Seminiferous Tubule Diameter and Cross-section Area (ST.D and ST. Ac)

No significant difference (P>0.05) in the diameter of the seminiferous tubule (St.D) and cross-sectional area of the seminiferous tubule (St. Ac) was observed across all treatment groups (figure 7 and 8).

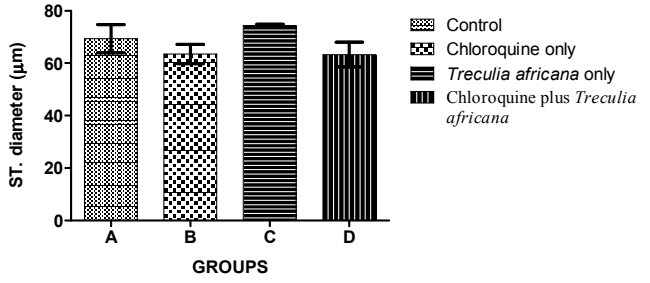

Figure 7. *Showing the diameter of the Seminiferous tubule in the treatment and control groups. *(P<0.05)-statistical significant.*

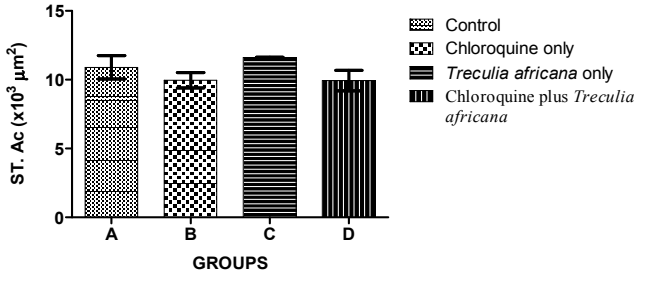

Figure 8. *Showing the seminiferous tubule cross sectional area (CSA) in the treatment and control groups. *(P<0.05)- statistical significant.*

3.4.2. Seminiferous Tubule Profile Per Area (ST.NA)

Seminiferous tubule profile per area reported A significant decrease (P<0.05) in group B compared to the control while none was observed in groups C and D compared to the control (figure 9).

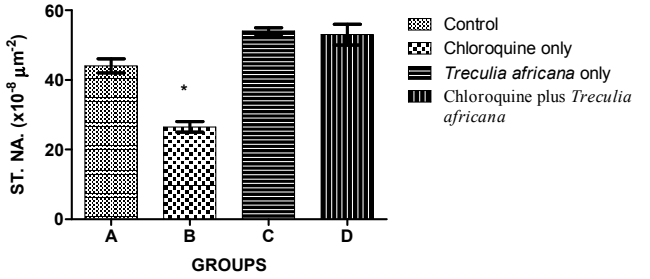

Figure 9. *Showing the seminiferous tubule profile per area in the treatment and control groups. *(P<0.05)- statistically significant.*

3.4.3. Numerical Density of Seminiferous Tubules (ST.NV)

Numerical density of the seminiferous tubules reported a significant decrease (P<0.05) in group B compared to control while group D significantly increased (P<0.05) when compared to the control group (figure 10).

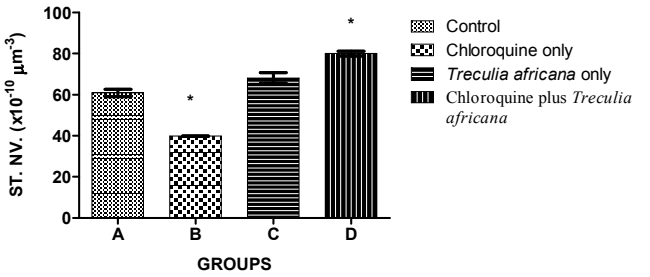

Figure 10. *Showing the Numerical densities of seminiferous tubules in the treatment and control groups. *(P<0.05)- statistical significant.*

3.4.4. Length Density of the Seminiferous Tubules

A significant decrease (P<0.05) in the length density of seminiferous tubules was observed in group B alone while groups C and D was at par when compared to the control (figure 11).

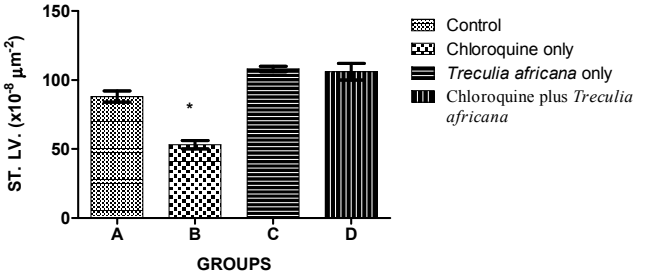

Figure 11. *Showing the Length densities of seminiferous tubules in treatment and control groups. *(P<0.05)- statistical significant.*

3.5. Histopathogical Ranking/Johnsen's Score

No significant difference (P>0.05) in Johnsen's Score was recorded in groups B and C while group D was at par with the control (figure 12).

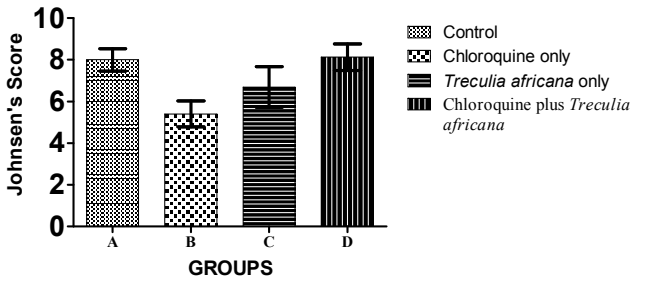

Figure 12. *Showing the Histopathological ranking between experimental and control groups.*

3.6. Histological Observations

Histological findings in group A showed a well defined testicular cytoarchitecture, semimiferous tubules and leydig cells (figure 13A). Group B showed degenerated seminiferous tubules, widened lumen with variable cells and reduced spermatozoa (figure 13B). Group C showed normal seminiferous epithelium lined inward by spermatogenic cells, well defined basement membrane and Lumen (figure 13C) while group D showed a well-defined seminiferous tubules consisting of well-defined basement membrane, normal seminiferous epithelium, leydig cell and spermatogenic cells (figure 13D).

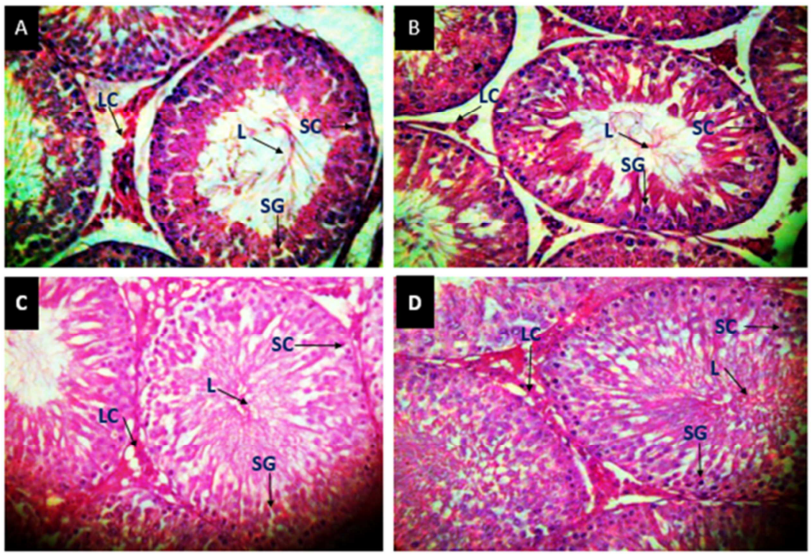

Figure 13. *(A-D): Photomicrographs of the testis in the adult male Wistar rats between treatment groups and the control.LC- Leydig cells, SG-Spermatogonia, SC- Sertoli cells, L-Lumen. Haematoxylin and Eosin stain X100 (A).*

3.7. Immunohistochemical Observations

Results in group A showed large populations of immunopositive cells (figure 14A). There was no expression of the marker at all in group B (figure 14B) while groups C and D showed little expression of immunopositive cells (figures 14C and D).

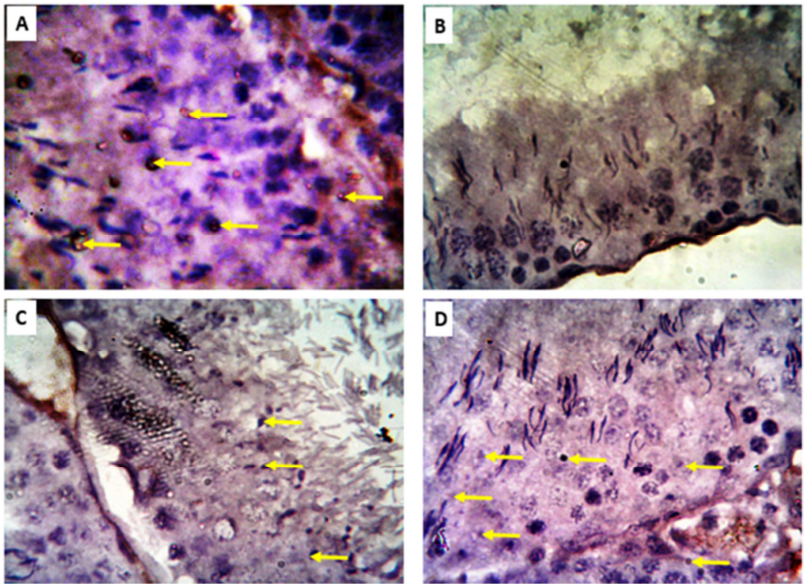

Figure 14. *(A-D): Showing the Photomicrographs of the testes of rats in control and treatment groups. The yellow arrow shows expression of immunopositive cells.*

4. Discussion

Chloroquine has anti-weight properties which resulted in *TASE* not having effects on the rat's body weight. Elgndy [5] reported correlation between decrease in body weight and chloroquine administration.

The drastic reduction in Sertoli (Figure 13B) and Leydig cells affected the process of spermatogenesis, hence the decrease in motility, viability and morphology of the testis. This corroborated previous reports by [5]. However the improvement recorded in (Figure 13D) may be as a result of the active phytochemicals present such as flavonoids, polyphenols and anthraquinones in the TASE which helped to remediate it. This was corroborated by [16] who reported that phytochemicals ameliorated testicular deficiencies.

In this study, the decrease in the sperm count reported was due to the interference between testicular tissue degeneration and the process of spermatogenesis. Thus leading to reductions in the amount of germinal epithelium and the number of matured sperm cells while the increase in sperm count may be due to the reparative effect of the active phytochemicals present like the flavonoid and polyphenols in the TASE [10]. These were in consonance with Arash et al. [17], who reported an increase in semen parameters in rats treated with flavonoids obtained from *TASE*.

The pathogenesis of male reproductive impairment and choloroquine-induced testicular toxicity may be a multi-factorial process. Its administration disrupts tubular and structural integrities of the testis by acting on the status of antioxidants, leading to antioxidants imbalance with subsequent development of oxidative stress, generation of free radicals and significant reduction in superoxide dismutase and hydrogen peroxide-decomposing enzymes such as catalase as well as increase in thiobarbituric acid-reactive substances due to accumulation of superoxide radical [18].

The decrease in testosterone levels could be attributed to a drastic reduction in Leydig cells, which was responsible for the production of the hormone. However, group D had an insignificant increase ($P<0.05$) (figure 6) and this may be due to the presence of flavonoids leading to intact leydig cells in that group of rats [19].

The decrease in luteinizing hormone (LH) levels and increase in the levels of follicle stimulating hormone (FSH) in this study, may be as a result of the physiological endocrine response to reduction in blood testosterone level; which resulted to a reactive elevation in the blood levels of gonadotropins and vice versa [20]. Another reason for the discrepancies in FSH and LH levels respectively may be due to idiopathic factors.

The abundant proliferative cells observed in the Immunohistochemical studies (Figure 13 C and D) was attributed to the active ingredients found in *TASE* while the group given only chloroquine had no expression of the proliferative marker which is an indication of the arrest of spermatogenesis at the level of differentiation from Type-A to Type-B spermatogonia. The immunohistochemical observations further points to the fact that chloroquine is indeed injurious to men's reproductive health due to the total absence of proliferative cells.

A significant reduction in sterological analysis in the number of seminiferous tubules profiles per area and length densities in group B (figures 9 and 11) was an indication of testicular morphological disruption reported in this study. Khodeary *et al* [21] reported that long-term administration of chloroquine phosphate in experimental animals caused severe degeneration of the seminiferous tubules and testicular tubular structures. However, non-significant increase observed in group C and D showed that TASE conferred some reparative advantages on chloroquine induced testicular toxicity (figures 8-11). The increase in numerical density observed further highlighted the ameliorative properties *TASE* had on testicular damage. However, these results shed light on the ameliorative properties of *TASE* on effects of chloroquine induced testicular damage thereby helping to restore the testicular histoarchitecture and structural integrity to a certain degree.

5. Conclusion

In conclusion, this study showed that chloroquine induced testicular damage resulted in male reproductive dysfunction via alterations in seminiferous tubules, semen analysis and hormonal levels. However, the administration of *TASE* helped to improve seminiferous tubule integrity. Therefore, we may suggest that TASE may be harnessed as a medicinal product to prevent male reproductive dysfunction and more research should be carried out.

Conflict of Interest

All the authors do not have any possible conflicts of interest.

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