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Morphological and Biochemical Evaluation of the Testes of Hyperglycemic Guinea Pigs Treated with *Cyperus Esculentus* Extract

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Abstract

In this study, we aimed to evaluate the effects of *Cyperus esculentus* extract on Testicular Morphology and Biochemistry post streptozotocin-treatment in Male Guinea Pigs. Twenty guinea pigs were divided into four groups containing five pigs each. Group A served as the negative control was treated with 5ml/kg body weight of normal saline orally, Group B were treated with a single dose of 65mg/kg body weight of streptozotocin (STZ) intraperitoneally, Group C were treated with a single dose of 65mg/kg body weight of STZ and daily treatment with 150mg/kg of CEE orally and Group D were treated with a single dose of 65mg/kg body weight of STZ and daily treatment with 150mg/kg of CEE orally for 20days. Morphological scores and biochemical evaluation were done. Results showed a significant decrease in testicular weight, number of spematogonia, Leydig cells and Sertoli cells, levels of SOD and CAT as well as a significant increase in the level of MDA after the administration of STZ alone when compared to the control. A significant decrease in the levels of testosterone and FSH as well as a decrease in the level of LH was observed in group B when compared to the control. However, the groups that were co-treated with the extract had a better parameters and histological profiles when compared to the positive control group. CEE has a dose-dependent ameliorative effect on STZ-induced testicular hormonal, oxidative and histomorphometric degeneration.

Keywords: hyperglycemia, infertility, testes, toxicity, Cyperus Esculentus, Guinea pigs, streptozotocin

1. Introduction

Conclusive results from numerous studies have cast doubt on the beliefs that Diabetes mellitus (DM) has minimal effects on male reproductive function in recent years. Male infertility is the inability of a male to successfully fertilize a fertile female during the course of 12 months of regular, unprotected sexual activity (WHO, 2017). An estimated 56% of child-bearing age infertile couples seek medical attention (Boivin et al., 2007).

Due to a decline in the spermatogenic process, roughly 50% of infertility cases are correctly attributed to the male spouse, favoring women somewhat in the statistics (Akunna et al., 2017). Reduced sperm motility and a rise in aberrant sperm morphology were found in studies on the frequency of infertility in DM male partners of infertile couples (Li, 2004). Recently, there has been growing concern about reproductive science because lifestyle tends to cause a lot of the diseases. Ageing, nutrition, exercise, coffee, a high scrotal temperature, hot water use, cell phone use, and even diabetes mellitus are among the culprits.

There are two main forms of diabetes mellitus: type 1 and type 2, each of which has a different pathogenesis and

etiology (Skyler, 2004). But there is one thing that undoubtedly unites the two, and that is hyperglycemia and all of the difficulties that go along with it (Abraira et al., 1995; Ohkubo et al., 1995).

Insulin Dependent Diabetic Mellitus is caused by absolute insulin deficiency due to autoimmune destruction of the pancreatic â-islet cells, while NIDDM typically results from a mix of insulin resistance and insufficient compensatory insulin release response, whereas diabetic mellitus is caused by absolute insulin shortage as a result of autoimmune destruction of the pancreatic â-islet cells (American Diabetic Association, 2012). The fundamental characteristic of DM is chronic hyperglycemia, which causes long-term harm, malfunction, and failure of several organs, affecting almost all body systems.

Numerous studies have linked diabetes with hypogonadism, retrograde ejaculation, impotence, and erectile dysfunction in both diabetic humans and animal models (Jain et al., 2014). Many organic foods, including fruits, vegetables, and general plants, have had their therapeutic properties researched (Jacks et al., 2007).

Of course, the use of alternative medicine in plants is quickly evolving (Ibrahim et al., 2009). Nigerians of northern descent grow the tiger nut (Cyperus esculentus) on a regular basis. It is a member of the Cyperaceae family and is indigenous to warm temperate and subtropical areas of the Northern Hemisphere. The somewhat sweet, nutty flavor of the tubers makes them appropriate for eating. A staggering amount of alkaloids, saponins, tannins, and vitamin E are still present in tiger nuts (Ekeanyanwu et al., 2010).

Its anti-microbial, anti-bacterial, cardiovascular, and anti-thrombotic properties have been described (Sofowora et al., 1993; Abano et al., 2011; Adejuyitan, 2009). It also plays androgenic roles (Malviya et al., 2002; Kamatenesi and Oryem, 2005). So, after treating male guinea pigs with streptozotocin (STZ), we sought to explore the effects of *Cyperus esculentus* extract (CEE) on Sperm Physiology, Testicular Morphology, and Biochemistry. To be more precise, our goal will be to:

i. Assess the impact of CEE and STZ on the testicular weight and volume of male guinea pigs.

ii. To assess the impact of CEE and STZ on the number of Leydig and Sertoli cells in the male guinea pigs' testicles.

iii. To assess the impact of CEE and STZ on male guinea pigs' testicular oxidative stress markers and reproductive hormones.

2. Materials and Methods

2.1 Tiger Nut Procurement and Aqueous Extraction

Fresh Tiger nuts were obtained from the nearby farmers market and was identified and authenticated at the herbarium department of the University of Abuja with the herbarium voucher number 284/34.

The nuts were washed, air dried, and cleaned of stains and dust. The nuts were finely processed into powder form. In 1000 cc of clean, warm water, 5g of dry TN was dissolved, mixed, and left for 48 hours. The residue from the extraction process was utilized to determine the dosage and yield residue percentage (Chukwuma et al., 2010).

2.2 Experimental Animals

Before the experiment began, twenty (20) adult male guinea pigs were obtained and given time to acclimate in a rat cage. They were given a rat pellet diet and unlimited access to water. Before and after the extract was administered, the guinea pigs were weighed and observed, respectively. Using an electronic analytical weight scale, the animals' weights were determined at the time of acquisition, during acclimatization, and at the conclusion of the experiment.

The guinea pigs were housed in industrial cages and kept at room temperature at the anatomy department's animal house at the university. Before the trial began, they were given a week to adjust to their new surroundings. They were given regular food and unlimited water to drink.

Moderately consistent environmental conditions were kept under control with good lighting and proper aeration.

Twenty guinea pigs were separated into four groups.

Streptozotocin (STZ) was administered intraperitoneally in a single dose of 65 mg/kg body weight to Group A, which served as the control group. Group B received daily treatment with 150 mg/kg of *Cyperus esculentus* extract and Group C received a single dose of 65 mg/kg body weight of STZ. Group D received a single dose of 65 mg/kg body weight of STZ and daily treatment with 150 mg/kg of CEE.

2.3 Animal Sacrifice and Sample Collection

Following a 12-hour fast, the guinea pigs were initially weighed before being sacrificed by cervical dislocation. To reveal the testes, the skin of the abdomen was delicately peeled off using forceps, scissors, and a scapula. Each animal's testicular weight was measured using an electronic analytical and precision balance. Using the

Archimedes principle and the water displacement method, the testicular volumes were measured. Each guinea pig's two testes were measured, and the average value was determined. Each animal had one testis preserved in Boun's solution for histological and morphometric examination. Each animal's remaining testes were kept in storage at -25°C for upcoming biochemical tests.

2.4 Morphometric Analysis

Leydig and Sertoli cells, as well as the diameter of the seminiferous tubules, were measured in a unit area of the testis. Two "vertical sections" from the polar and equatorial areas were sampled for each testis (Qin & Lung, 2002), and a systematic random method was used to obtain an unbiased numerical estimation of the following morphometric parameters (Gundersen & Jenson, 1987):

2.4.1 Diameter (D) of Seminiferous Tubules

For each animal, the diameter of seminiferous tubules with round or nearly round profiles was measured. A mean, D, was then calculated by averaging the two diameters, D1 and D2 (Perpendicular to one another).

Only when D1/D2 is less than 0.85 will D1 and D2 be used. Number of Leydig and Sertoli cells in a unit area of testis (NA): This was established by applying the Gundersen-proposed unbiased counting frame (1977). With this frame, we not only counted the profiles that were entirely inside the frame but also all the profiles that had any portion inside the frame as long as they didn't touch or overlap the full-drawn line, the exclusion edges, or their extension.

3. Biochemical Parameters

3.1 Estimation of Lipid Peroxidation (Malondialdehyde)

The thiobarbituric acid reactive substances (TBARS) method of Buege and Aust was used to colonimetrically determine the amount of lipid peroxidation in the supernatant (1978). Malondialdehyde (MDA), a byproduct of lipid peroxidation, is a key component of TBARS. In a nutshell, 2 ml of the TBAA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl, and 15% TCA) were added to 0.1 ml of tissue in TrisHCl buffer, pH 7.5, and the mixture was then placed in a water bath for 15 minutes before cooling. At 535 nm, the absorbance of clear supernatant was measured in comparison to a reference blank. Malondialdehyde's molar absorptivity, which is 1.56 x 105 M-1 cm-1 and is represented as nmol/mg protein, was used to calculate concentration.

3.2 Assay of Superoxide Dismutase (SOD) Activity

According to Rukmini et al's description, superoxide dismutase activity was assessed using the Winterbourn et al. (1975) method (2004). The assay's basic idea was founded on SOD's capacity to prevent the reduction of nitro-blue tetrazolium (NBT).

The reaction mixture contains 0.1 ml of enzyme samples, 0.05 ml of 0.21 mM riboflavin, 0.1 ml of 1.5 mM NBT, and 0.05 ml of 0.01 mM methionine in addition to 2.7 ml of 0.067 M phosphate buffer, pH 7.8. By putting these tubes in a box with a 15W fluorescent lamp for 10 minutes, air aluminum foil was used to provide uniform illumination of the tubes.

There was also a control group without the enzyme source. At 560nm, the absorbance was measured. The amount of enzyme necessary to prevent NBT from being reduced by 50% under the given circumstances was determined to be one unit of SOD.

Enzyme activity was measured in units per mg of protein.

3.3 Assay of Catalase (CAT) Activity

The Aebi technique was used to measure the catalase activity (1983). In a cuvette containing 1.9 ml of 50 mM phosphate buffer, pH 7.0, supernatant (0.1 ml) was pipetted. Addition of 1.0 ml of freshly made 30% (v/v) hydrogen peroxide to the reaction (H2O2). Utilizing spectrophotometry, the rate of H2O2 oxidation was calculated from variations in absorbance at 240 nm.

Enzyme activity was measured in units per mg of protein.

3.4 Assay of Testosterone (TT) Activity

The enzyme immunoassay method, which is based on the idea of competitive binding between TT and TT-horseradish peroxidase conjugate for a fixed amount of rabbit anti-TT, was used to measure the amounts of ttestosterone in homogenate supernatant (Tietz, 1995). In a nutshell, TT standards, controls, samples (blood sera and supernatants of testicular homogenates), TT-horseradish peroxidase conjugate reagent, and rabbit anti-TT reagent were incubated at 37°C for 90 minutes with goat anti-rabbit IgG-coated wells.

The wells were cleaned after unbound TT peroxidase conjugate was removed. After the addition of tetramethylbenzidine and an incubation period, a blue color developed. With the addition of 1N hydrochloric acid, the color development was stopped, and the absorbance was measured spectrophotometrically at 450 nm.

Plotting the standard's concentration versus time yielded a standard curve the absorbance and TT concentrations calculated from the standard curve.

3.5 Assay of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH)

The assays were carried out in accordance with the Amballi et al. adapted process (2007). The blood that was gathered and placed in simple containers was briefly allowed to clot. To achieve separation, each sample was centrifuged at 1000 rpm for 10 min. Each time, the serum was divided into aliquots, labeled, and stored at -200C. To estimate hormone levels using enzyme immunoassay (EIA), one aliquot of each specimen was taken at a time in order to prevent repeated freezing and thawing. This was done in accordance with the World Health Organization (WHO) matched reagent programme protocol (manual) for EIA kits (protocol/version of December 1998 for LH, FSH).

The NIADDK-NIH provided the children (USA).

3.6 Statistical Analysis

Data from the study's findings were expressed as mean SEM (standard error of mean). Statistical Package for Social Sciences (SPSS) version 20.0 was used for the statistical analysis. One-way Analysis of Variance (ANOVA) was used to compare the means of the treated groups and the control group, and Dunnett T3 post-hoc testing was used to determine whether there was a statistically significant difference. Statistics were considered significant for values under 0.05.

4. Result

4.1 Effect of Cyperus Esculentus Extract on Testicular Weight, Spermatogonia, Leydig and Sertoli Cells

A significant decrease ($p<0.05$) in testicular weight, number of spematogonia, Leydig cells and Sertoli cells was observed in Group B when compared to the control (Group A) (**Table 1**). A dose dependent significant increase ($p<0.05$) was observed for the number of spermatogonia in Group C. A dose significant increase ($p<0.05$) was also observe for the testicular weight, number of spematogonia, Leydig cells and Sertoli cells in Group D.

Table 1. Effect of *Cyperus esculentus* extract on testicular weight, Spermatogonia, Leydig and Sertoli cells of Male Wistar Rat

Groups	**Testicular weight (g)**	**Spermatogonia**	**Leydig cell**	**Sertoli cell**
A (5ml/kg saline)	1.56±0.18	9.03±0.35	27.26± 1.90	9.06±0.83
B (45mg/kg STZ)	0.36±0.12*	0.95 ±0.54*	14.2±1.00*	5.70±0.50*
C (45mg STZ+150mg/kg CEE)	0.40±0.10	1.22 ±0.70**	13.26±2.65	6.20±0.95
D (45mg STZ+300mg/kg CEE)	1.00±0.43**	1.19 ±0.68**	18.50±5.98**	9.30±3.27**

Note: * and ** represent significant difference at $p<0.05$ when compared to group 1 and group 2 respectively.

4.2 Effect of Cyperus Esculentus Extract on Enzymes of Oxidative Stress

There was a significant decrease ($p<0.05$) in the levels of SOD and CAT as well as a significant increase ($p<0.05$) in the level of MDA after the administration of STZ in Group B when compared to the control (**Table 2**). A dose dependent significant increase in SOD ($p<0.05$), an increase in CAT as well as a significant decrease in MDA ($p<0.05$) was observed in Group C. A dose dependent significant decrease ($p<0.05$) in the level of SOD, a decrease in the level of CAT as well as a significant decrease ($p<0.05$) in the level of MDA was observed in group D.

Table 2. Effect of *Cyperus esculentus* extract on oxidative stress Markers

GROUPS	**SOD (U/mg pro))**	**MDA (nmol/mg pro)**	**CAT (U/mg pro)**
A (5ml/kg saline)	23.83±2.53	0.72±0.32	12.0±1.66
B (45mg/kg STZ)	11.63±1.26*	2.08±1.04*	7.60±0.62*
C (45mg STZ+150mg/kg CEE)	15.06±3.61**	1.23±0.15**	8.56±2.56
D (45mg STZ+300mg/kg CEE)	14.56±3.00**	1.16±0.45**	8.36±0.85

Note: * and ** represent significant difference at $p<0.05$ when compared to group 1 and group 2 respectively.

4.3 Effect of Cyperus Esculentus Extract on Testosterone, Follicle Stimulating Hormone and Luteinizing Hormone

A significant decrease ($p<0.05$) in the levels of testosterone and Follicle stimulating hormone as well as a decrease in the level of luteinizing hormone was observed in Group B when compared to the control **(Table 3).** A dose dependent increase which was not significant was observed in the levels of testosterone, follicle stimulating hormone and luteinizing hormone in Group C. A dose dependent increase which was significant ($p<0.05$) was further observed in the levels of testosterone, follicle stimulating hormone and luteinizing hormone in Group D.

Table 3. Effect of *Cyperus esculentus* extract on Testosterone, follicle stimulating hormone and luteinizing hormone

GROUPS	Testosterone (ng/ml)	FSH (ng/ml)	LH (ng/ml)
A (5ml/kg saline)	2.50±0.60	0.73±0.10	8.54±1.39
B (45mg/kg STZ)	1.40±0.30*	0.43±0.15*	7.60±.60
C (45mg STZ+150mg/kg CEE)	1.60±0.62	0.50±0.26	7.86±1.52
D (45mg STZ+300mg/kg CEE)	2.16±0.92**	0.86±0.20**	9.86±8.33**

Note: * and ** represent significant difference at $p<0.05$ when compared to group 1 and group 2 respectively.

5. Histology

A proliferation of the spermatogenic series into matured spermatozoa was observed in the seminiferous tubular lumen (L) of the control (**Group A**). The basement membrane was intact for group A and Leydig cells were present in the interstitium. There was deterioration in the proliferation rate of the spermatogenic series in STZ treated pigs (**Group B**). Detrioration in the interstitium was further observed in the STZ treated pigs. Leydig cells were absent in the interstitium of STZ treated rat testis. An improvement in the proliferation of the spermatogenic series into matured spermatozoa in the seminiferous tubular lumen was observed in the low dose CEE treated pigs (**Group C).** A restoration in the interstitium of the testis of low dose CEE-treated pigs was observed also in Group C. An even further improvement in the spermatogenic series into matured spermatozoa was observed in the high dose CEE treated pigs (**Group D**). Leydig cells were observed to be present in the interstitium of the high dose CEE treated pigs.

6. Discussion

Male infertility has become a common condition among many couples in recent times. Studies have shown that 50% of cases infertility among couples is attributed the males probably as a result of challenges with spermatogenesis (Akunna *et al.,* 2017). Modifiable lifestyle factors such as nutrition, physical activity, use of mobile telephones, consumption of caffeine and alcohol, frequent use of hot water, high scrotal temperature, diabetes mellitus (DM) etc. play a crucial role in infertility challenges and this has led to several studies in this field.

DM is a condition that has a multidisciplinary approach in its management. While physicians have adopted Insulin therapy and the use of oral anti-hyperglycaemic agents in its management, there been lots of challenges in using these drugs including cost, dosage, administration techniques, availability, side effects etc. Hence researches into alternative therapy in medicinal plants have become very necessary. Although several medicinal plants have been used in the treatment of male infertility arising from different etiologies, only a few of them have been investigated to identify specific roles they play in ameliorating infertility challenges secondary to DM. In this light the present study treated subsets of pigs with *Cyperus esculentus* extract (low dose and high dose) for 20days after administering a single dose of streptozotocin (40mg/kg b.wt.) and there was a significant ($p>0.05$) improvement in the levels of Testosterone (TT), follicle stimulating hormone (FSH) and leuitenizing hormone (LH) in Group D (Table 3) which had a higher dosage of CEE and a significant ($p>0.05$) decrease in level of testosterone and follicle stimulating hormone but no difference ($p>0.05$) in level of leuitenizing hormone of pigs treated with STZ when compared to Group A (Table 3). The increase may be attributed to the effect of CEE. Also, the variance in the level of TT, FSH and LH observed in Groups C and D may be attributed to a dose dependent role of CEE. However, the reduction in the level of TT, FSH and LH seen in Group B were in accordance with the reports of Sexton and Jarow (1997), Schoeller *et al.* (2012), Ahmed & Kurkar (2014) and Youssef & Zidan (2016).

The main function of the testes is the production of sperm and testosterone. Testosterone is the major male sex hormone, produced by the Leydig cells, which are located between the seminiferous tubules (Hsu *et al*., 2003).

Spermatogenesis, anabolic growth and secretion are primarily the functions of testosterone. Follicle stimulating hormone (SFH) functions in Induction and maintenance of spermatogenesis. FSH along with testosterone, is necessary for maintaining normal sperm count and function (Orlowski & Sarao, 2019; Dwyer & Quinton, 2019). Lutenizing hormone causes the Leydig cells of the testes to produce testosterone and also stimulates synthesis and secretion of testosterone (Nedresky & Singh, 2019). The results suggest that STZ may affect testosterone release through the hypothalamo–hypophyseal–gonadal axis, which plays an important role in the development and regulation of some of the body's systems. Studies have shown that the interference in the hypothalamus secretion of GnRH by opioids leads to consequent decrease in LH and FSH release from the pituitary and TT from the testis (Abdellatief *et al*., 2015). Surprisingly, STZ seem to have no effect on the level of LH in our study. In the present study, Group B recorded a significant ($p<0.05$) decrease in value of SOD and CAT while showing an increase in MDA in contrast to Group A the control group. A significant ($p<0.05$) difference was also observed in the values of SOD and MDA for both Groups C and D which received CEE when the control group. However, there was no ($p>0.05$) significant difference in activity level of CAT in groups treated with CEE when compared with control group. This report is in accordance with those of Ahmed & Kurkar (2014) and Ibrahim & Salah-Eldin (2019).

Superoxide dimutase (SOD) is an essential component of the cellular antioxidant defense system. It is the first line of defense against oxidative stress with dismutation of superoxide radicals to H_2O_2 and molecular oxygen, catalase (CAT) converts hydrogen peroxide to oxygen and water, a decrease in both superoxide dimutase (SOD) and catalase (CAT) by oxidative stress would lead to the high production of reactive oxygen species like H_2O_2 which reacts with various intracellular compounds such as DNA, lipids and proteins.

Malondialdehyde (MDA) is the end product of lipid peroxidation. The elevated level of malondialdehyde (MDA) is an index of the extent of lipid peroxidation and oxidative stress. This suggests that the toxic effects of STZ at a cellular level might be explained by lipid peroxidation (LPO) (Ibrahim & Salah-Eldin, 2019). Reports have shown immature spermatozoa and leukocytes as the major source of ROS in the male reproductive system (Agarwal *et al.,* 2014; Leclerc *et al.,* 1997). Also, a cascade of events is said to occur when the level of ROS exceeds the seminal antioxidant defense system, the result is damage in sperm DNA, fragmentation of mitochondrial DNA, alterations in the sperm parameters and ultimately infertility in males. (Ahmed, 2005). It can also cause a decreased ATP levels, thereby affecting the kinematics of spermatozoa. (Akunna *et al.*, 2017; Ibrahim & Salah-Eldin, 2019). In agreement with these reports and to further confirm the ameliorative effects of CEE on reproductive dysfunction secondary to DM, the morphological and histological parameters were also investigated.

The present study revealed a significant ($p<0.05$) decrease in testicular weight, number of Spermatogonia, Leydig and Sertoli Cells in pigs treated with STZ in comparison with control group (Group A). However, in comparing Group B (STZ) with Group D (CEE), there was a significant ($p<0.05$) increase in testicular weight, number of Spermatogonia, Leydig and Sertoli Cells. Some reports have it that when some testicular structures such as seminiferous tubules and Leydig cells are distorted due to decrease in layer of germ cells it could lead to a reduction in testicular mass Mostafa *et al.* (2016). Sakamoto *et al.*, 2008 and Arai *et al*. (1998) in their study also suggested that testicular volume and testicular function could be correlated and this was also observed in the present study. Lafuente *et al.* (2001) had suggested that the observed reduction in testicular function may be due to STZ toxicity on the hypothalamic-pituitary-gonadal axis. In agreement with previous reports (Abdolahnejad *et al.,* 2009; Long *et al.,* 2015; Ukwenya *et al.*,2020), the present study revealed that the group treated with STZ showed that the seminiferous tubules degenerated and were atrophied, the germinal epithelium was vacuolated and there was absence of late germ cells. There was low testicular weight, also degeneration of spermatogenic cells and absence of sperm bundles. In this light, the present study demonstrates that treatment with CEE improves the histomorphometric parameters of the testes, shows a near normal testicular architecture with numerous spermatozoa radiating towards the lumen and an elevated germinal epithelium with an evidence of intact interstitial cells and Sertoli cells as well as improved testicular weight. Thus, this ability marks CEE an excellent therapeutic choice for the management of male infertility secondary to DM. This is also in corroboration with previous reports by Udogadi and Onyenibe (2019).

7. Conclusion

Dietary based therapies may be potent alternative for managing and preventing infertility cases secondary to diabetes mellitus. The result of the present study revealed that STZ is testiculotoxic as shown by the oxidative, hormonal and histomorphometric evidences. Also, CEE has an ameliorative effect on STZ-induced testicular hormonal, oxidative and histomorphometric degeneration. However, the potency of CEE on STZ-induced testicular degeneration is dose-dependent as revealed in this study. Thus, CEE may be used as a therapeutic agent or a dietary choice for the management of male infertility arising from complications of DM.

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Appendix

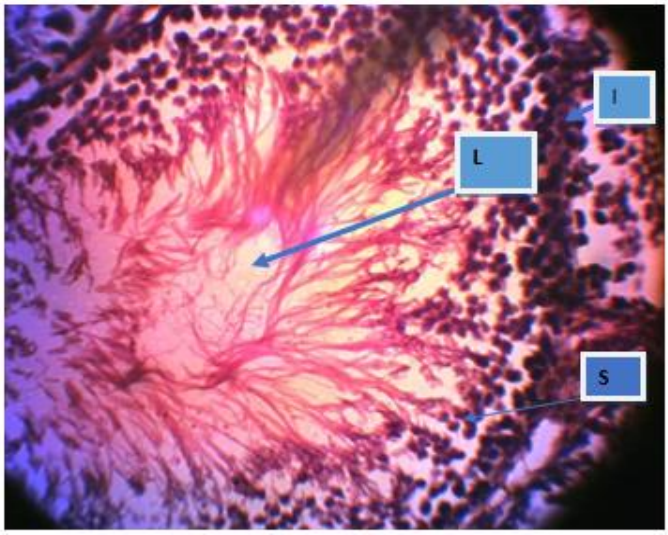

Figure 1. (Control) Group A: L= Lumen, S= Spermatids, IC= Interstitium

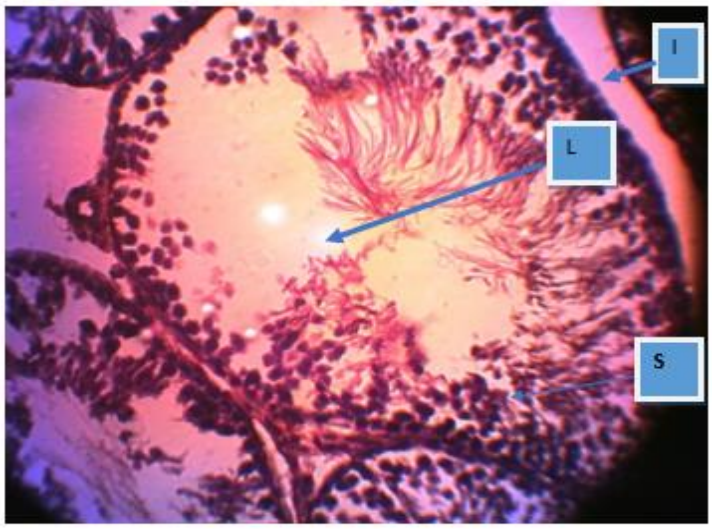

Figure 2. Group B: ST= Seminiferous tubule, S= Spermatids I= Interstitium

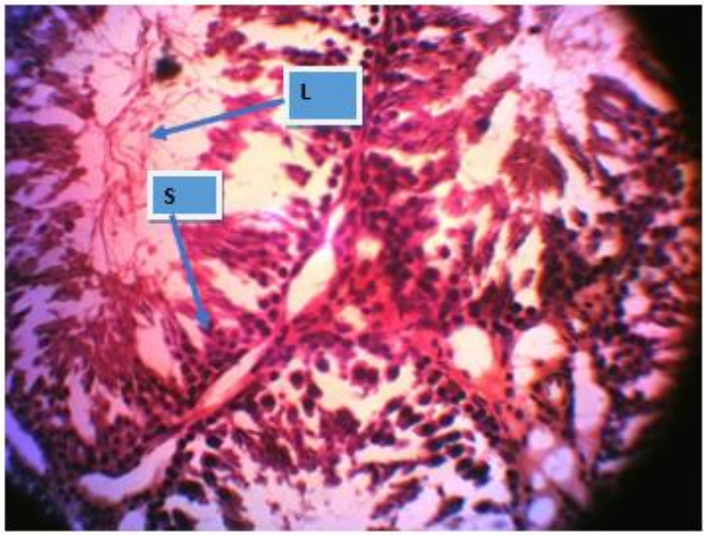

Figure 3. Group C: ST= Seminiferous tubule, S= Spermatids

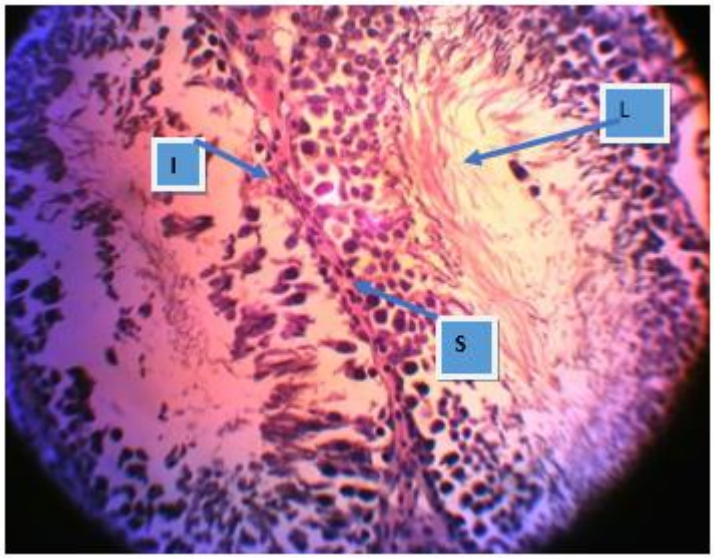

Figure 4. Group D: ST= Seminiferous tubule, S= Spermatids