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Effects of Streptozotocin-induced Diabetes Mellitus on theTestes of Wistar Rats

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Abstract
Twenty-one Wistar rats weighing 200 - 250 mg were divided into three groups- the control, saline and the diabetic groups, each comprising of seven rats per group. They were used to assess the effects of streptozotocin-induced diabetes on testes of Wistar rats. All the animals including the control group received feed mash and water ad libitum, the saline group received equal volumes of physiological saline intraperitoneally while the diabetic group was treated with intraperitoneal 55 mg/kg of streptozotocin. The effects of these treatments on total sperm count, sperm motility, percentage live:dead sperm and on testicular and epididymal morphology of the Wistar rats were assessed. There was significant difference in the total sperm count ($P<0.005$) between the diabetic group ($24.5\pm3.7 \times 10^6$) and control ($55.8\pm4.7 \times 10^6$) and in the motile sperm count ($P<0.001$) between the diabetic group ($15.2\pm2.3 \times 10^6$) and control ($50.2\pm4.3 \times 10^6$). There was no significant difference ($P>0.05$) between the total ($53.0\pm4.2 \times 10^6$) and motile ($46.3\pm3.7 \times 10^6$) sperm count in the saline group compared to control. The diabetic group had greater numbers of dead and morphologically abnormal spermatozoa as further evidenced from the histology of the specimens. The findings of this investigation confirms the adverse effects of hyperglycaemia on testicular and epididymal functions in diabetic state.
Keywords: Streptozotocin, Diabetes Mellitus, Testes, Wistar rats.

Introduction
Diabetes mellitus is a metabolic disorder that poses a worldwide challenge. It affects people of different age-groups. It has been described as a heterogenous metabolic disorder characterized by hyperglycaemia resulting from defective insulin secretion, resistance to insulin action or both (1). Type II diabetes is often regarded as maturity on-set diabetes affecting mainly the elderly. The incidence of type I and type II diabetes mellitus is increasing, affecting youths of reproductive age group (2). This situation render the youths vulnerable to complications of diabetes such as infertility (3). Diabetes mellitus has also been reported to have induced testicular dysfunction (4). Male infertility is a common threat nowadays and it has increased rapidly partially because of hyperglycaemia (5). Roy *et al.,*(6) reported the role of Naringenin in diabetes-induced testicular damage and reiterated Sexton and Jarrow (7) as well as Akkoc *et al.,* (8) definition of diabetes mellitus as a degenerative disease with alteration in carbohydrate homeostasis that affects male reproductive function at multiple levels particularly the endocrine control of spermatogenesis, spermatogenesis itself or by impairing penile erection and ejaculation.

Streptozotocin is used in experimental diabetes. It is an antibiotic derived from streptomyces achromogenes. Streptozotocin-injected rats is said to possess many characteristics resembling those of insulin-dependent diabetic mellitus in humans such as hyperglycaemia, ketonuria and hyperlipidaemia (9). According to Roy *et al.* (6), it causes energy deprivation and apoptotic damage to beta cells of the pancreas via alkylation or breakage of DNA strands. This leads to increase in the activity of poly-ADP- ribose synthetase, an enzyme that depletes the NAD^+ in beta cells. How this would affect normal testicular and epididymal cytoarchitecture and functions in the Wistar rats is the focus of this study.

Methodology
Animals and Intervention
Experimental procedures involving the animals and their care were conducted in conformity with International and Institutional guidelines for the care of laboratory animals in Biomedical Research, as promulgated by Canadian Council of Animal care (10). Further, the animal experimental models used were in conformity to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding Principles in the care and use of Animals (11, 12).

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The animals were procured from a standard breeding stock and housed in well ventilated wire wooden cages in the animal facility of the department of Anatomy, University of Benin, Benin City. The rats were maintained under standard natural photoperiodic condition of 12 hr of light alternating with 12 hr of darkness (i.e. L:D;12:12) with room temperature of between 25 to 26^0C and humidity of 65±5%. They were given water *ad libitum* and unrestricted access to feeds obtained from Bendel feeds and flour mill, Ewu; in Edo State, Nigeria. They were allowed to acclimatize for three weeks (21 days) before the commencement of the experiments. The experimental animals were categorized into the following groups:

I. **Control Group:** Received Feed mash and water only *ad libitum* throughout the duration of treatment before sacrifice at the end of the twenty-fourth week.
II. **Saline Group:** This group served as negative control. They received equivalent (quinine) volume of physiological saline before sacrifice at the end of the twenty-fourth week.
III. **Diabetic Group:** This group of rats was subjected to overnight fasting. Diabetes mellitus was induced via intraperitoneal (i.p.) injection of a single dose of streptozotocin (55 mg/kg) which induced diabetes by rapid depletion of beta cell mass causing reduction in insulin release and hyperglycaemia. Streptozotocin was dissolved in a freshly prepared 0.01 M citrate buffer at pH 4.5. The streptozotocin-injected animals were given 1 ml of 50 % dextrose in 1:1 dilution with distilled water intramuscularly to prevent initial hypoglycaemia related mortality observed within the first 12-24 hours post-induction. Diabetes was confirmed using the 'Fine test' blood glucose monitoring kit (glucometer), with strips manufactured by infopia Co. Ltd; Korea. Rats with blood glucose levels of 250 mg % or more were considered diabetic and used in the experiment. The range of blood glucose of all the diabetic rats was maintained between 300-450 mg % using titrated dosage of insulin throughout the period of experiment before their sacrifice. This group was induced with diabetes on the twelfth week and the rats were sacrificed at the end of the twenty-fourth week.

Animal sacrifice and collection of sample:

The rats at the time of sacrifice were weighed and then anaesthesized by placing them in a closed jar containing cotton wool soaked with chloroform anaesthetic. The abdominal cavity was opened up through a midline abdominal incision to expose the reproductive organs. The testes were excised and trimmed of all fats. Each testis was weighed using an electronic analytical and precision balance (Mettler Pm 4800 Delta RangeR). Scooped caudal epididymal sperm was mixed with physiological saline and collected in a sterile bottle for assessment of sperm parameters.

Sperm Count

This was done using the new improved Neubauer's counting chamber (Heamatcytometer). To achieve this, 0.9 ml of physiological saline solution was mixed with 0.1 ml of the epididymal fluid. The counting chamber was cover slipped, filled with sperm fluid and placed under a binocular light microscope using an adjustable light source. The ruled part was focused and the number of spermatozoa was counted in five 16-celled squares. The total sperm cells were added, and multiplied by 10^6 and expressed as (X) × 10^6/ml, where X is the total number of sperm cells in the five 16-celled square (13). The degree of sperm progressivity was assessed following the modified procedure of Saalu *et al.,* (12).

The one step eosin: nigrosin staining technique for Live: Dead Sperms:

This procedure was carried out according to the staining solution for the one step technique containing 0.67% eosin Y and 10% nigrosin as adopted by Mortimer (14, 15). Semen samples were liquefied at 37°C for 30 min before analysis. Approximately equal volumes of semen and stain were mixed. Using an automatic pipette, 50 µl of undiluted and well mixed liquefied semen was mixed in a ceramic well with equivalent volume of the eosin-nigrosin staining solution (50 µl). The suspension was incubated for 30 seconds at room temperature (20°C). Then, a 10 µl droplet was transferred with automatic pipette to a labelled microscope slide where it was smeared by sliding a cover slip in front of it. Two smears were made from each sample. The smears were air dried and examined directly. From an original concentration of 1,000,000 spermatozoa in 0.5 ml obtained from each group, 10 µl of the semen was diluted in 49,990 µl of formal saline to obtain the required concentration of 0.1 µl using an automatic pipette. From this, an aliquot of 0.5 ml was sampled to obtain 200 spermatozoa for each group that was assessed at a magnification of 1000× under oil immersion. Spermatozoa that were white (unstained) were classified as live and those that showed any pink or red colouration were classified as dead, with the sole exception for sperm with a slight pink or red appearance restricted to the neck region ('leaky necks'), which were assessed as live (15).

Histology: Routine histological preparation

The organs were harvested and fixed in bouin's fluid for 24 hours, after which it was processed by automated processor and stained with haematoxylin and eosin staining techniques (16). Photomicrographs were at magnification x40. Effects on testicular and epididymal cyto-architecture such as spermatogenesis, presence or

absence of Sertoli cells in the seminiferous tubules, changes in the interstitial cells, presence of spermatozoa or immature germ cells in the epididymis, e.t.c., were noted.

Data Analysis

Data were presented as Mean ± SEM. Means separation was by Duncan multiple range test (17, 18) and significant differences between the means were determined by student t-test at ***($p<0.001$)[a], ($p<0.005$)[b], ($p<0.05$)[c].***

Results

Sperm Parameters

The data on total sperm count and % motility, viscosity, volume, pH and % live: dead sperm were analyzed and the values obtained for each group, compared to control are shown in the table. From the results, a constant volume of the sperm (0.5ml) was used. The pH value for the semen samples collected was 6.0. The results of the total and motile sperm count from the table revealed as follows: For the control group, mean total sperm count was 55.8±4.7 $x10^6$/ml, mean motile sperm count was 50.2±4.3 $x10^6$/ml and % motile sperm was 80.2±10.8. For the saline group, total sperm count was 53.0±4.2$x10^6$/ml, motile sperm count was 46.3±3.7$x10^6$/ml, and % motile sperm was 87.7±2.2. The diabetic group had mean total sperm count as 24.5±3.7$x10^6$/ml out of which 15.2±2.3$x10^6$/ml were motile, which is 60.7±4.2 % motility. The results of the total sperm count in the diabetic group was significantly different *($P<0.005$)* from the control and the motile sperm count as well *($P<0.001$)*, but there was no significant difference *($P>0.05$)* between the saline group and the control in both the total and the motile sperm count.

The degree of sperm progressivity was rapidly motile and forward (**) in the control and the saline groups, but more sluggish, curved and less forward in progressivity (*) in the diabetic group. Complete immotility (#) was not observed in any of the groups.

Percentage ratio of the live: dead sperm evaluated in: control group was 82:18, saline group was 81:19 and in the diabetic group was 64:36. Live sperm are unstained with the eosin-nigrosin stain, remaining pale or white while dead spermatozoa take up the stain as pink or red.

The histological out-look of the treatments such as saline and diabetes mellitus on the testicular and epididymal tissues are shown in Figs. 1 - 4. The control sections of the testes essentially showed the lumen of the seminiferous tubules with clusters of spermatozoa and spermatogenic cell series in progression from the basement membrane towards the adluminal compartment leading to release of matured spermatozoa into the lumen. The testicular interstitium were essentially normal containing the interstitial cells of Leydig. The testicular and epididymal sections of the saline treatment show normal progression of the spermatogenic germ cell series and normal testicular interstitium. The sections of the testes and epididymis from the diabetic group had various testicular lesions such as seminiferous tubules with atrophic changes, necrosis, detachment of the basement membrane from the seminiferous epithelium, disrupted seminiferous epithelium and arrested spermatogenesis. Inflammatory changes and few interstitial cells (of Leydig) were noticed in the testicular interstitium. The epididymis showed luminal depletion of spermatozoa, discontinuity of epithelial lining, focal necrosis and fibrosis.

Table 1: Effects of Streptozotocin-induced Diabetes on Sperm Parameters

Groups	Total Sperm Count $X10^6$/Ml	Motile Sperm Count $X10^6$/Ml	% Motile Sperm	Progressivity	% Live: Dead Sperm
Control	55.8±4.7	50.2±4.3	80.2±10.8	**	82:18
Saline	53.0±4.2	46.3±3.7	87.7±2.2	**	81:19
Diabetic	24.5±3.7[b]	15.1±2.3[a]	60.7±4.2	*	64:36

*** Means with alphabetic remarks are significantly different from control: ($p<0.001$)[a], ($p<0.005$)[b], ($p<0.05$)[c]. Vertical comparisons only.**

Control: Received feed mash and water only

Saline: Received equivalent volume of normal saline

Diabetic: Streptozotocin-induced diabetic group

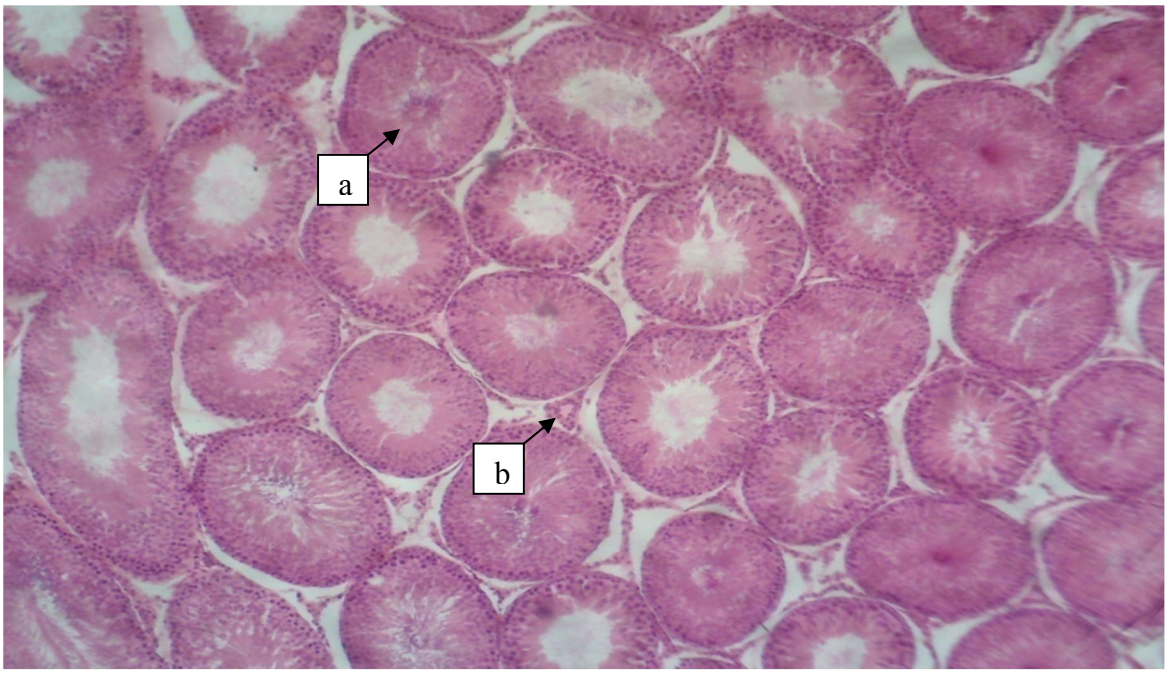

Fig 1a: Section of the control C3 testis (a) normal seminiferous tubule containing germ cells of the spermatogenic series (b) normal testicular interstitium with Leydig cell [H&E x40]

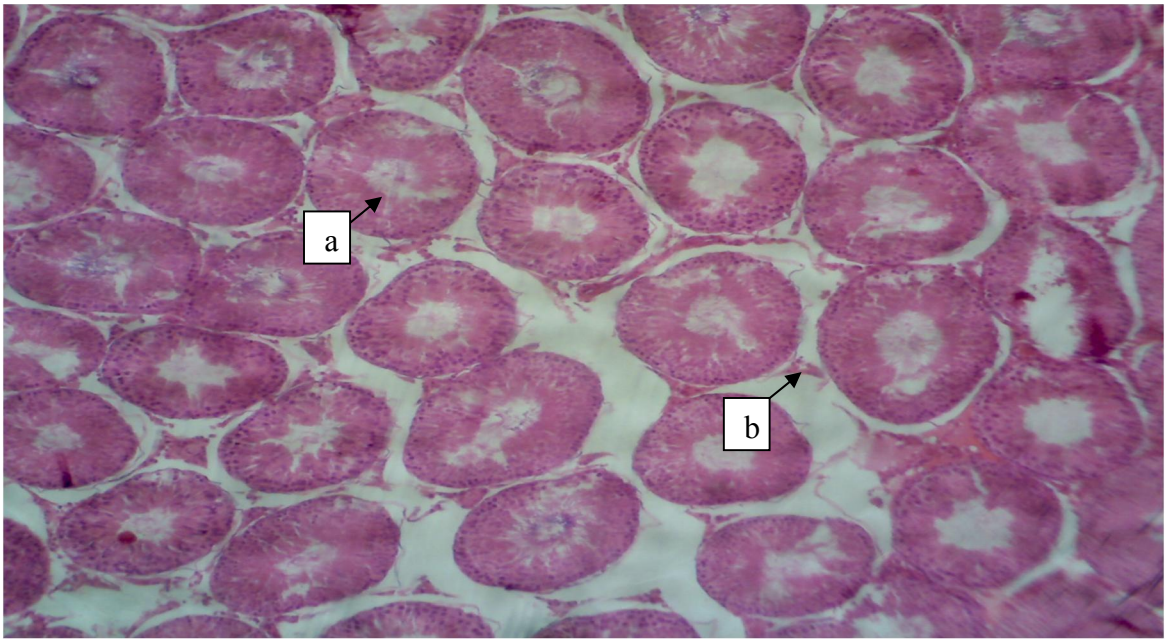

Fig 1b: Section of saline S4 testis (a) normal seminiferous tubule containing germ cells of the spermatogenic series (b) normal testicular interstitium with Leydig cell and mild oedema [H&E x40]

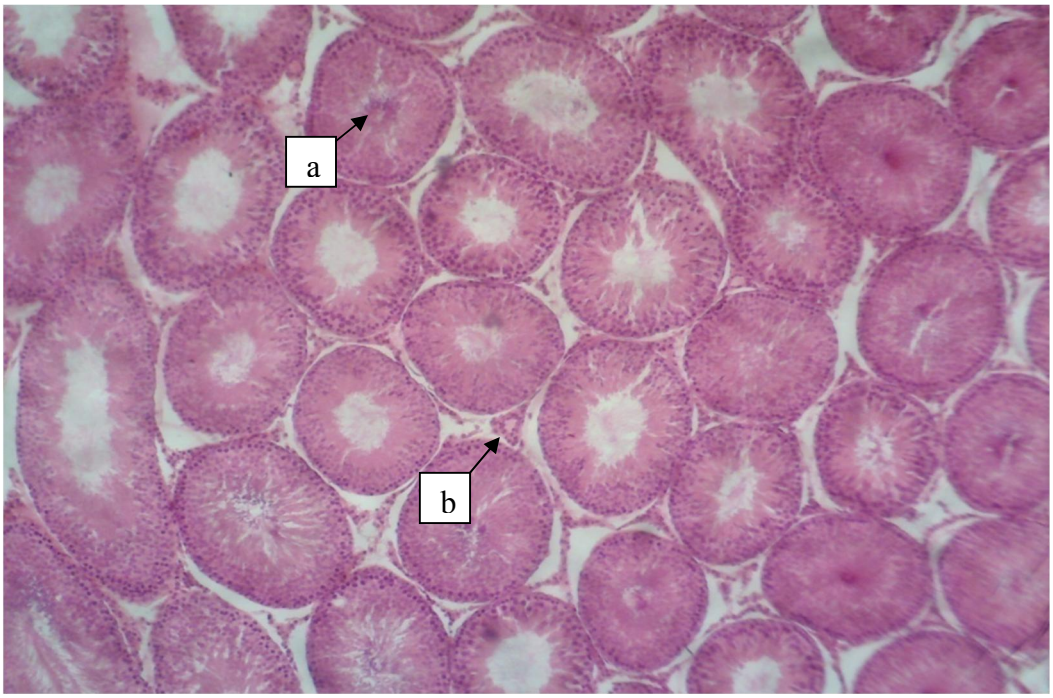

Fig 2a: Section of the control C3 testis (a) normal seminiferous tubule containing germ cells of the spermatogenic series (b) normal testicular interstitium with Leydig cell [H&E x40]

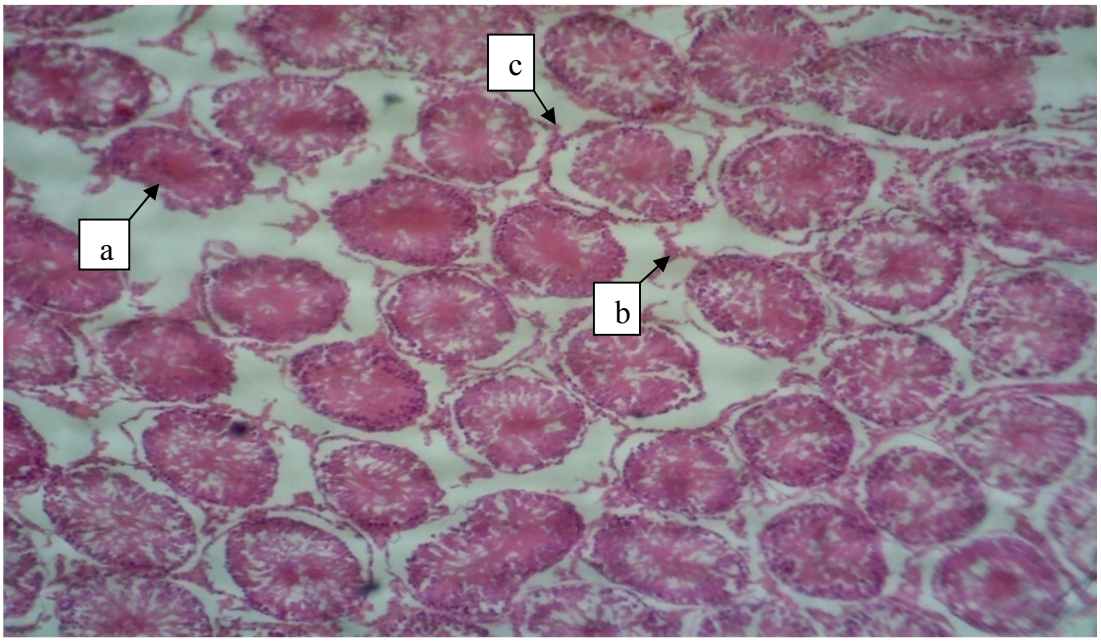

Fig 2b: Section of diabetic group DM3 testis (a) seminiferous tubule with atrophic tubules and degenerative changes (b) testicular interstitium with necrosis and few Leydig cells (c) detached basement membrane from the seminiferous epithelium [H&E x40]

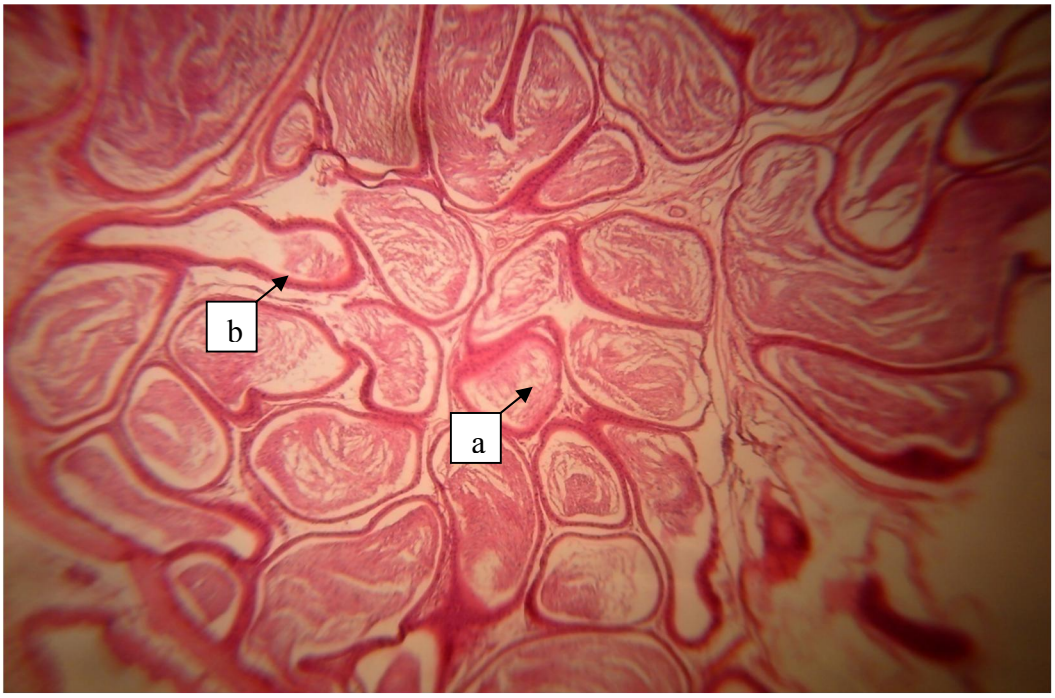

Fig 3a: Section of the control C3 epididymis (a) lumen containing spermatozoa stores (b) normal lining of the epididymis [H&E x40]

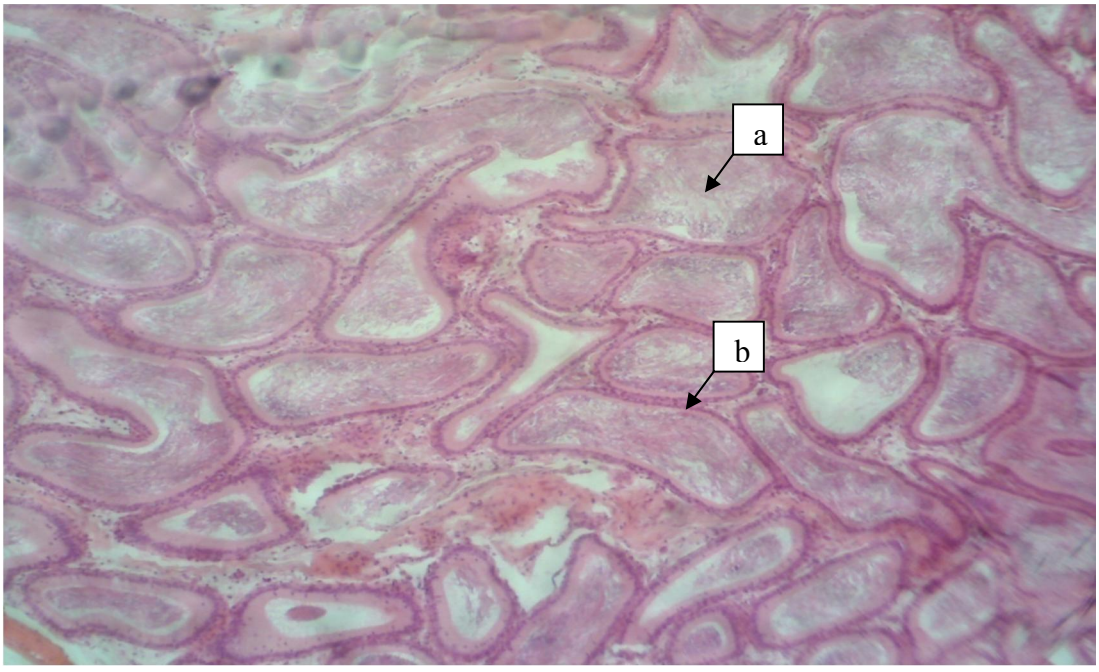

Fig 3b: Section of the saline S4 epididymis (a) lumen containing spermatozoa stores (b) normal lining of the epididymis [H&E x40]

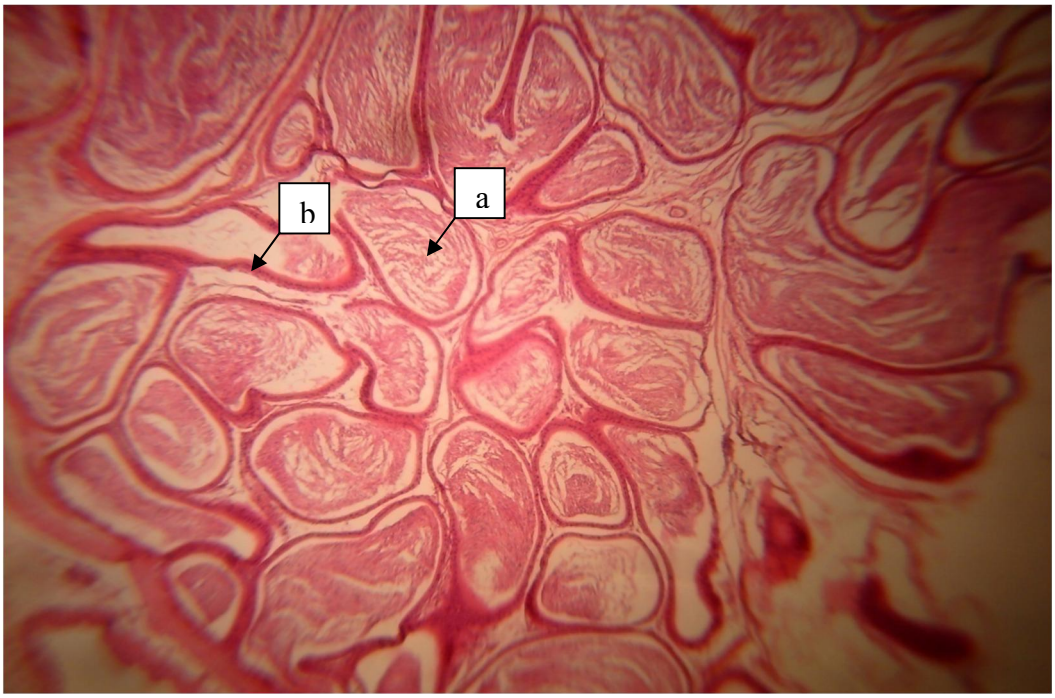

Fig 4a: Section of the control C3 epididymis (a) lumen containing spermatozoa (b) normal epithelium of the epididymis [H&E x40]

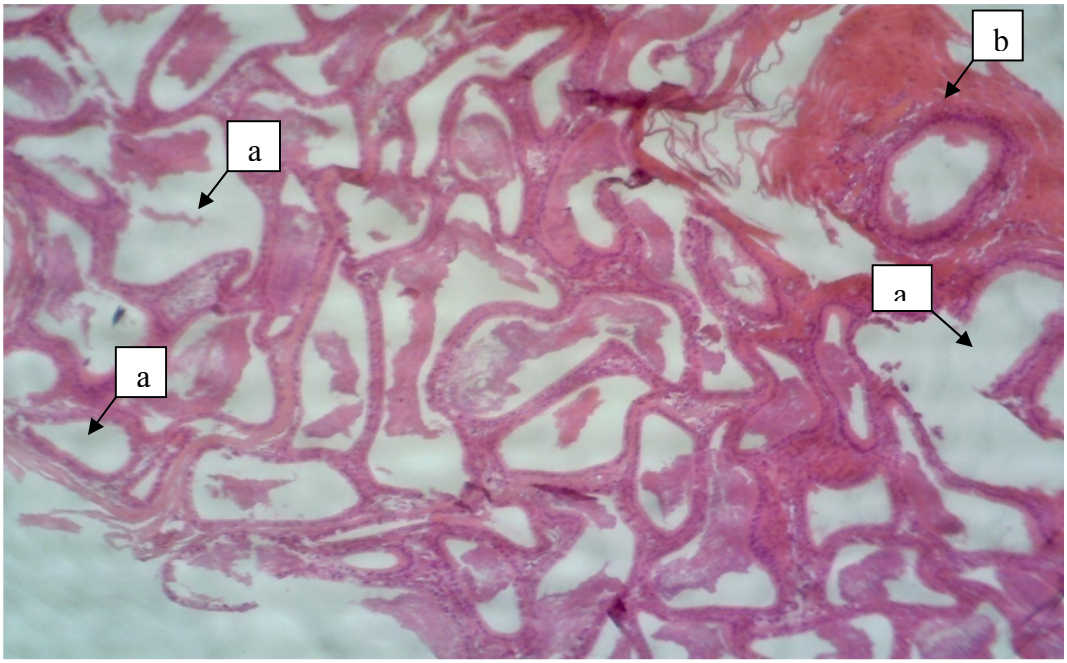

Fig 4b: Section of diabetic group DM3 epididymis (a) empty lumen or scanty spermatozoa in the lumen (b) fibrotic changes around the epithelial lining of the epididymis [H&E x40]

Discussion

The results of total sperm count in the diabetic group was significantly different from control ($p<0.005$), and the motile sperm count in the diabetic group was also significantly different from control ($p<0.001$). There was a greater percentage of dead sperm and more morphologically abnormal forms in the diabetic group, compared to control. These findings of reduced total sperm count and motile sperm count in diabetes-induced testicular damage in this study is consistent with previous report by Akkoc *et al.* (8), on how diabetes could cause impairment of testicular functions. Amaral *et al.,* (19) had attributed impairment of spermatogenesis in hyperglycaemic condition as in streptozotocin-induced diabetes to be as a result of decreased testicular ATP levels leading to a compromised adenylate energy change. This position is also supported by Roy *et al.,* (6). The observation of atrophic seminiferous tubules, altered spermatogenesis and arrest as well as inflammatory changes in the testicular interstitium and few interstitial cells (of Leydig) are evident to the observed fertility challenge seen in diabetes. This is because the epididymal storage of matured spermatozoa is thereby affected as demonstrated in this study from the observed luminal depletion of spermatozoa. Aitken *et al.,* (20) had also remarked on the role of excess reactive oxygen species and free radicals and their adverse effects on sperm motility and fertility. It is therefore noteworthy that hyperglycaemia truly has deleterious effects on the testis, affecting energy levels, sperm count, motility and morphology. The implication of this is that the increasing incidence of diabetes globally might potentiate a corresponding increase in infertility challenges, especially the male factor-induced type. The management of diabetes-induced testicular damage and thus male infertility is poor and still a serious challenge. This is because in diabetic state, hyperglycaemia caused by the rapid depletion of beta cell mass by streptozotocin (21) results in oxidative stress that can result in DNA damage of the testis (19). Roy *et al.,* (22) posit that oxidative stress in streptozotocin-induced diabetic rats can affect the functions of the Sertoli cells which provides nutrients and regulatory factors to the developing germ cells in the seminiferous epithelium (23). Furthermore, oxidative stress affects mitochondrial functions by causing the release of cytochrome C and caspase activation which can lead to apoptotic damage[24]. These possible mechanisms provides explanation to our findings in this study, similar to the significant reduction in sperm parameters like sperm motility, sperm count and sperm viability observed in a related study (19). This calls for the need to source and identify agents with anti-oxidant property that can prevent or protect against diabetes-induced testicular damage if the current trend must be arrested and pave way for the achievement of the objectives outlined in the millennium development goals.

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