

Reproductive Parameters in Streptozotocin-Induced Diabetic Male Wistar Rats: Beneficial Role of *Basella Alba* Aqueous Leave Extract

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Abstract

Background: Diabetes mellitus (DM) is widely reported to have adverse effect on most systems of the body. This study investigated the effects of DM on male reproductive parameters and possible role of aqueous leave extract from *Basella alba* in ameliorating such effects.

Methods: Male rats (n = 40) aged 8-10 weeks were randomly divided into 4 equal groups: Healthy Control (HC), Diabetic Control (DC), Healthy Treatment (HT) and Diabetic Treatment (DT). Diabetes was induced in animal by using (STZ single intraperitoneal injection of streptozotocin (STZ), (55 mg/kg). Treatment was oral via gavage HC and DC groups received 0.5 ml/100 g normal saline daily via oral gavage, HT and DT groups received 200 mg/kg plant extract daily for 4 weeks. Fasting blood sugar and body weights were recorded weekly. Animals were sacrificed upon completion of treatments and tissues were collected for further analysis.

Results: There was significant decrease in weights of the body, testis, and epididymis in DC compared to HC rats ($P < 0.05$). Relative testicular weight was significantly increased in both DC and DT compared to HC groups ($P < 0.0001$). Sperm concentration, viability and morphology, were all significantly reduced in DC versus HC rats ($P < 0.0001$), but improved in DT versus DC ($P < 0.05$). Histopathological examination showed degenerative changes in DC specimens that were alleviated in DT rats.

Conclusion: Aqueous extract of *Basella alba* plays a major role in ameliorating male reproductive complications due to streptozotocin-induced diabetes mellitus.

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Introduction

Diabetes mellitus (DM) has been described as a metabolic disease that is characterized by hyperglycemia due to failure of insulin secretion, inappropriate insulin action or a combination of both anomalies. The two major types are insulin dependent or type 1 diabetes mellitus (T1DM) and non-insulin dependent or type 2 diabetes mellitus (T2DM), both of which are associated with disturbances in carbohydrate, protein and fat metabolism (1). Diabetes is often a consequence of either destruction of beta-islet cells resulting in insulin deficiency (T1DM) or a failure of the hormone secretion to exert its effects on target organs due to insulin resistance (T2DM) (2).

Streptozotocin (STZ) is one of the chemical agents commonly used to induce different types of DM in various animal models due to its toxic effect on the insulin producing beta-islet cells of the pancreas. The metabolic characteristics and severity of DM induced by STZ is dependent on a number of factors, including the dosing regimen of STZ, strain and gender of the animal (3).

DM was reported to affect 177 million people worldwide in the year 2000, with the figure projected to reach 300 million people by the year 2025 (4). However, by 2011, the International Diabetic Federation reported that, 366 million people were already affected by DM globally and the incidence was expected to rise to 522 million by the year 2030 (5). These alarming projections, in addition to the challenges associated with conventional drug treatments led to current research trends where various medicinal plants are studied for their potential effects in the treatment of DM. The focus is often directed towards the ability of these plants to lower blood sugar and improve general well-being of diabetic

patients, with little or no attention to whether or not specific systemic complications of DM are reversed by these plant agents.

Basella alba is one such plant that have been reported to lower blood sugar and improve general wellbeing in diabetic rats (6). It is a green vegetable plant that is originated from India (7), but is also grown throughout the tropics as a perennial plant and in warmer temperate regions as an annual crop. Its thick, semi-succulent heart-shaped leaves have a mild flavour and mucilaginous texture. The common names include; Ceylon spinach, Malabar spinach or Indian spinach, and it is a branched, climbing plant (8). Among many other uses, the plant has been reportedly used to alleviate weak libido in men suffering from subfertility in some parts of Cameroon (9). In the southern part of Nigeria, *Basella alba* leaves are commonly prepared as vegetable sauce which serves as condiment to most of the common staple food. Other systemic effects of this plant that have been reported include its potential as hematinic, antidiarrheal, anticonvulsant, anti-inflammatory, antifungal and analgesic agent (8).

DM is reportedly complicated by a wide variety of male reproductive dysfunction ranging from reduced libido to infertility and impotence (10). Reports on the impact of the disease on sperm parameters and semen quality are rather inconsistent, with discrepancies regarding the actual parameters affected and extent of the effects (11).

The mechanism by which *Basella alba* lowers blood sugar is not known and whether or not this anti-hyperglycemic effect translates to reversal of certain complications of DM is yet to be demonstrated in any study. This present work was targeted at investigating a possible role

for the aqueous extract of *Basella alba* at reversing some reproductive complications of DM in male Wistar rats.

Materials and Methods

Preparation of Plant extract

Fresh leaves of *Basella alba* were collected from various humid locations in south western Nigeria. The leaves were washed and then air-dried at room temperature for a period of three to four weeks. The plant was earlier identified and authenticated at the Department of Botany, University of Ibadan, Nigeria with voucher number (UIH-22391).

Aqueous extract was prepared as described by Iloki-Assanga et al., 2015 (12) with slight modification. Briefly, the dried leaves were grinded into fine powder and 100 g of this was added to 1000 ml of distilled water at 100°C and the mixture was maintained at that temperature in water bath for a further 5 minutes. It was subsequently removed from the heat and allowed to infuse for a further 30 minutes before being filtered. The filtrate was freeze-dried using LyoDry Grande freeze dryer, LSG60 (Bristol, UK) to form a powdery extract which was dissolved in normal saline and administered to the rats by oral gavage.

Animals and study design

Ethical approval for the study, (reference number; CPUT/HWS-REC 2015/A04) was granted by the Health and Wellness Sciences Research Ethics Committee (HWS-REC) of the Cape Peninsula University of Technology, Cape Town, South Africa. Forty (40) male Wistar rats, aged 8-10weeks were given free access to food (standard rat chow) and water unless when fasted prior to fasting blood sugar (FBS) sample collection. They were subjected to standard atmospheric

conditions with a twelve hours light/dark cycle. Animals were randomly divided into four groups (n = 10) and treated as follows; Healthy control (HC) and Diabetic control (DC) animals received normal saline at 0.5 ml/100g body weight daily, and Healthy Treatment (HT) and Diabetic Treatment (DT) rats were given the plant extract at an oral dose of 200 mg/kg body weight daily. Treatment in all four groups was administered by gavage via a metal endotracheal tube. The maintenance and care of experimental animals throughout the study complied with National Institutes of Health guidelines for the humane use of laboratory animals.

Induction of diabetes mellitus

Diabetes was induced in rats from the diabetic control and diabetic treatment groups through a single intraperitoneal injection of STZ, 55 mg/kg after an overnight fast. STZ was dissolved in ice cold citrate buffer (0.1 M) at a pH of 4.5 and prepared fresh, immediately before administration. Animals were allowed access to food and water afterwards and fasting blood sugar was recorded seventy-two hours later to confirm diabetes. Rats were considered diabetic and included in the study only when FBS was above 11.1 mmol/L (200mg/dL) (13).

Measurement of FBS and weight

FBS was recorded weekly in all four groups from tail capillary blood, by pricking the tip of the tail with a sterile lancet to express one or two drops of blood. This was applied to the glucometer (ONETOUCH® Ultra2) strip and FBS determined. Weight was recorded weekly using a portable electronic balance.

Sample and organ collection

All animals were euthanized after four weeks of treatment. Blood was collected via cardiac puncture into serum clot activator tubes (VACUETTE®), centrifuged at 4000rpm for 10min at 4°C and serum stored at -80°C for hormonal assays. The right testes and epididymis were removed, weighed and caudal epididymal spermatozoa were isolated for immediate analysis, while left testes and epididymis were preserved for histological studies. Relative organ weight was calculated by dividing the organ weight by the animal's total body weight.

Sperm isolation

The caudal epididymis was dissected out and freed of all fat and excess tissues. 0.5 cm of the distal end was then cut off, placed in 1ml of HAMS solution in a petri dish, chopped into four smaller pieces and incubated at 37°C for 1 minute to allow the spermatozoa to swim out before evaluation.

Sperm concentration and motility

Both the concentration and motility of the spermatozoa were determined by computer-aided sperm analysis (CASA), using the Sperm Class Analyzer, version 5.0 (SCA®, Microptic, Barcelona, Spain).

One minute after the onset of incubation of the epididymis in the medium (HAMS solution), 2 µl of the medium was removed with a pipette close to the edge of the hazy portion (i.e. the cloud of sperm swimming out of the epididymis). Subsequently, one 2 µl chamber of a standard count eight chamber slide (Leja® Netherland) was filled and placed on the heated (37°C) microscope stage and analysed under X40 magnification. Nikon E200 microscope was used with phase '2' setting and spermatozoa fields visualized on camera

(Basler® A312fc) at a frame rate of 25 fps (Frames per second). Minimum of 1000 spermatozoa from at least five different fields were evaluated per sample.

Sperm viability

Sperm viability was determined by the Eosin/Nigrosin (E/N) dye exclusion staining technique. To the sperm suspension, we added eosin and nigrosin in a 1:2:3 ratio and mixed in 1.5 µl Ependorf tube. A drop (10 µl) of this mixture was placed on a microscope slide and a thin smear prepared and allowed to air dry. After twenty four hours of air-drying, a cover slip was mounted with DPX and allowed to further dry prior to evaluating it with a light microscope using X100 magnification. The percentage viability was determined by evaluating 100 spermatozoa per slide and recording the number of live cells. Live spermatozoa were unstained (white), while dead spermatozoa took up the eosin stain and appeared pink/red.

Sperm morphology

The percentage of morphologically normal spermatozoa was determined after staining the spermatozoa with Sperm Blue® according to the technique described by Van Der Horst and Maree (14). A thin smear was made from the sperm suspension (10 µl) and the slide was left to air dry for twenty-four hours. The slide was subsequently immersed slowly in the sperm blue staining solution for sixty seconds after which it was placed at an angle of 60° - 80° for ten seconds to allow for draining of the excess stain. Finally the slide was gently immersed in distilled water (3-6 seconds), drained and left to air dry. Dry slides were cover slipped using DPX and evaluated on the Sperm Class Analyser®. Nikon E200

microscope was used with a blue filter and phase 'A' setting under X60 magnification. Only spermatozoa which do not overlap with each other or with background staining were considered for evaluation (fifty spermatozoa per slide).

Reproductive Hormones

Testosterone levels in all the serum samples was determined by radioimmunoassay technique using testosterone rat/mouse enzyme-linked immunosorbent assay (ELISA) kit (DEV9911) according to the manufacturer's specifications (Demeditec®).

Both serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels were determined using the MILLIPLEX® MAP Rat Pituitary Magnetic Bead Panel (RPTMAG-86K), employing Luminex® xMAP® technology.

Histopathological Analysis

Histological study of the testes and epididymis was conducted by fixing samples from each rat in 10% buffered formalin immediately after excision. These were washed, trimmed and processed by embedding in paraffin. The specimens were sectioned at 5 µm thickness and stained with Hematoxylin and Eosin (H&E stain). Slides were examined microscopically according to the method of Luna (15) under the guidance of a histopathologist who was blinded to the study.

Statistical analysis

Data was analysed using Graphpad Prism version 5, and values were expressed as mean ± SEM. Differences between group means were determined by one-way analysis of variance (ANOVA). Bonferroni post-test used to compare data

from all four groups. A value for $P < 0.05$ was considered to be statistically significant.

Results

Fasting blood sugar

The FBS of diabetic animals (DC = 18.69 ± 1.37 mmol/L, DT = 17.74 ± 1.34 mmol/L) were significantly elevated ($p < 0.0001$) compared to that of the healthy animals (HC = 4.48 ± 0.15 mmol/L, HT = 4.51 ± 0.15 mmol/L) at the onset of the study. The FBS of DC animals significantly increased over the four weeks treatment period (18.69 ± 1.37 mmol/L vs. 24.71 ± 1.14 mmol/L, $p < 0.001$). After 4 weeks of treatment, the FBS of DT animals receiving *Basella alba* was significantly reduced compared to their initial values (10.71 ± 0.41 mmol/L vs. 17.74 ± 1.34 mmol/L, $p < 0.0001$), as well as significantly reduced compared to FBS levels in diabetic (DC) animals that only received normal saline (10.71 ± 0.41 mmol/L vs. 24.71 ± 1.14 mmol/L, $p < 0.001$). FBS remained unchanged in both control and treated healthy rats (Fig. 1a).

Body weight

The body weight of both HC (291.9 ± 11.26 g vs. 333.6 ± 13.36 g) and HT (272.4 ± 4.43 g vs. 306.1 ± 4.58 g) animals increased significantly ($p < 0.05$) during the 4 weeks experimental period. The percentage weight gain was 14% for HC and 12% for HT animals.

A significant loss ($p < 0.001$) in body weight was observed in DC (268.0 ± 16.79 g vs. 182.8 ± 11.75 g) and DT (251.3 ± 10.42 g vs. 183.5 ± 5.50 g) animals during the same period of time. DC and DT animals lost 32% and 27% weight respectively (Fig. 1b).

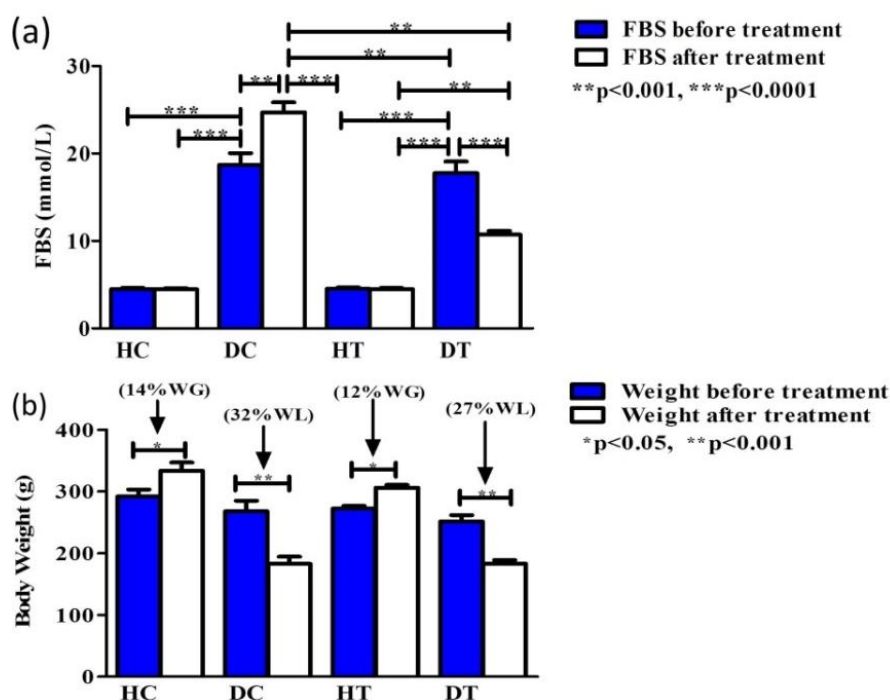


Fig. 1 (a) Fasting blood sugar (FBS) before commencement of treatment and after four weeks of treatment. (b) Body weight changes before and after treatment [WG=Weight gain, WL=Weight loss]

Organ and relative organ weights

The testicular weights (right) of DC and DT animals were significantly lower ($p < 0.001$) when compared to that of both HC and HT animals. *Basella alba* treatment had no effect on the testicular weight of healthy animals (HC vs. HT, not significant) or diabetic animals (DC vs. DT, not significant) as depicted in Fig. 2a. Upon calculating the relative testicular weights (i.e. Right testis weight/Body weight), it was observed that these ratios were significantly higher ($p < 0.001$) in DC and DT animals compared to HC and HT animals. Yet again, no differences were observed in ratios between *Basella alba*

treated animals and their respective controls (i.e. HC vs. HT and DC vs. DT) (Fig. 2b).

Similarly, epididymal weights (right) of DC and DT animals were significantly lower ($p < 0.0001$) when compared to both HC and HT animals. However, the epididymal weight of *Basella alba* treated diabetic animals (DT) was significantly higher ($p < 0.005$) compared to their control (DC) animals (Fig. 2c). The relative epididymal weight (Right epididymis weight/Body weight) was also significantly higher ($p < 0.05$) in DT animals when compared to DC. No differences were observed among the relative epididymal weights of DT, HT and HC animals (Fig. 2d).

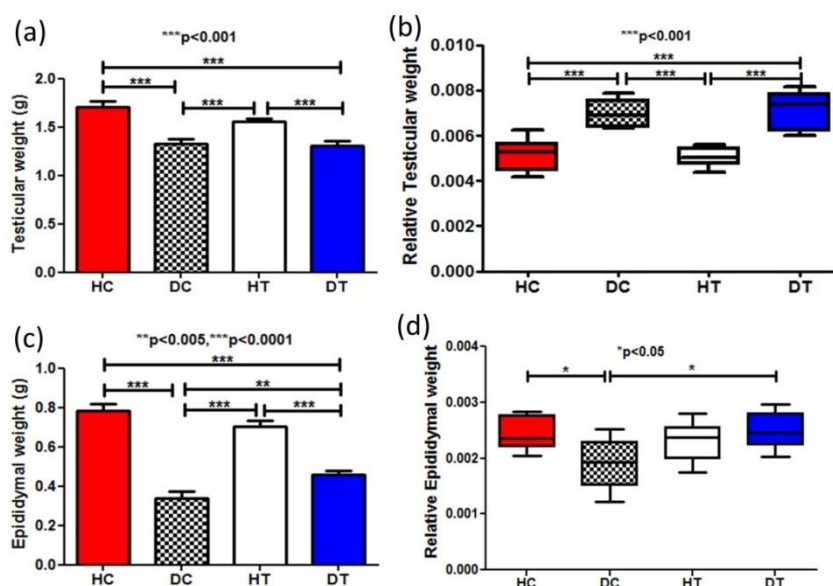


Fig. 2 (a) Average weights of right testis after treatment. (b) Average relative testicular weight after treatment. (c) Average weights of right epididymis after treatment. (d) Average relative testicular weight after treatment.

Sperm parameters

Sperm concentration was significantly lower ($p < 0.05$) in the DC rats when compared to the HC, but significantly higher ($p < 0.05$) in DT animals compared to DC. Four weeks of *Basella alba* treatment did not affect the sperm concentration of healthy animals (i.e. HC vs. HT, not significant). No significant differences were observed in the total motility of spermatozoa among all 4 groups. The percentage of viable spermatozoa was significantly lower in the diabetic control ($p < 0.001$) and diabetic treatment ($p <$

0.05) groups when compared to the healthy control. However, the viability values were significantly higher ($p < 0.001$ and $p < 0.05$) in the healthy and diabetic treatment groups respectively when compared to the diabetic control group. The percentage normal morphology was also significantly lower ($p < 0.001$) in the diabetic control group compared to healthy controls and significantly higher ($p < 0.001$ and $p < 0.05$) in healthy treatment and diabetic treatment groups respectively in comparison to diabetic control (See Table 1).

Table 1. Sperm parameters in control and treatment groups after four weeks of experiment

Parameters	Healthy control	Diabetic control	Healthy treatment	Diabetic treatment
Concentration (million/ml)	4.50 ± 0.49	2.23 ± 0.57 *	3.88 ± 0.88	4.52 ± 0.63 a
Motility (%)	74.30 ± 7.23	53.30 ± 10.37	70.90 ± 6.48	69.50 ± 6.88
Viability (%)	70.13 ± 2.0	31.67 ± 1.63**	70.43 ± 1.73b	50.50 ± 1.73*a
Normal Morphology (%)	62.96 ± 2.71	41.67 ± 2.33**	68.00 ± 2.80 b	53.67 ± 2.39 a

Values expressed as mean ± SEM for 10 rats per group. Statistical significance were represented as;

* $P < 0.05$, ** $P < 0.001$ vs. Healthy control

^a $P < 0.05$, ^b $P < 0.001$ vs. Diabetic control

Serum testosterone, LH and FSH

Serum testosterone levels were significantly increased in the diabetic control group when compared to the remaining three groups ($p < 0.001$) as shown Fig. 3a. A similar trend was also observed for serum LH with significantly higher levels in

DC animals compared to all three of the other groups ($p < 0.0001$) (Fig. 3b). There was no significant difference in the levels of FSH among all four experimental groups as illustrated in Fig. 3c.

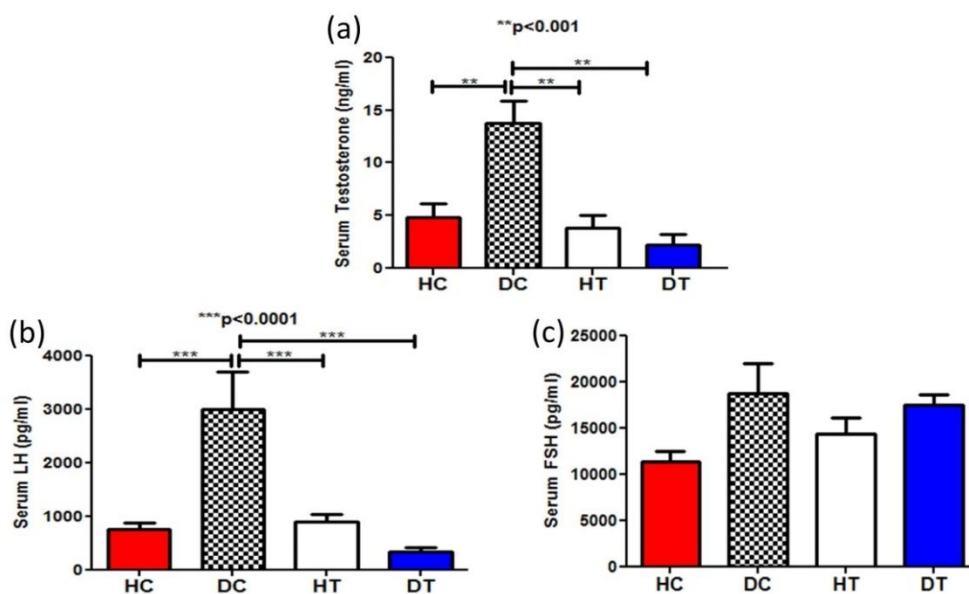


Fig. 3 (a) Serum testosterone levels after treatment. (b) Serum Luteinizing hormone (LH) levels after treatment. (c) Serum Follicle stimulating hormone (FSH) levels after treatment.

Histopathology of the testes and epididymis

Histopathological examination of the testes of HC and HT rats showed no visible lesions (Fig. 4a and c) when compared to DC specimens which displays patchy arrangement of severe germinal tissue necrosis of the seminiferous tubules and moderate edema of the testicular interstitium. The affected

tubule sections are interspersed between normal non-necrotic sections (Fig. 4b). The testes of diabetic rats treated with aqueous extract of *Basella alba* (DT) showed nearly normal histology, with mild to moderate interstitial congestion (Fig. 4d).

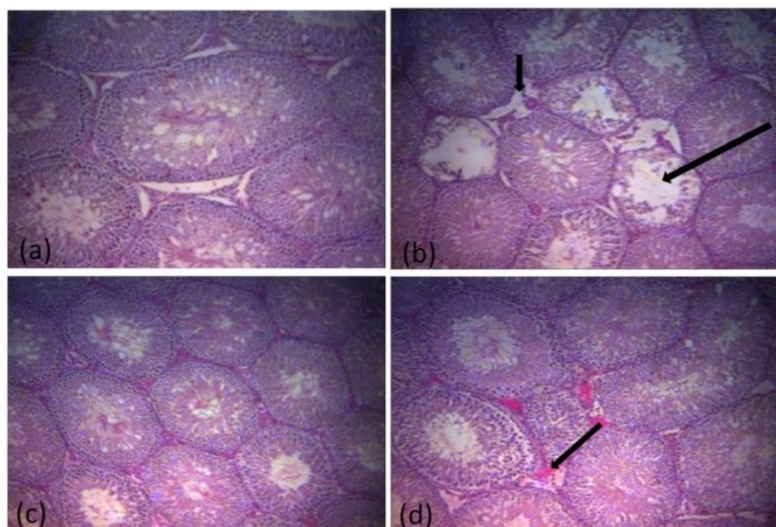


Fig. 4 Histopathological Photomicrographs (H & E, x100) showing; (a) Testes of a healthy control (HC) rat with normal seminiferous tubules. (b) Testes of a diabetic control (DC) rat with severe germinal tissue necrosis of the seminiferous tubules (Long arrow) and moderate edema of the testicular interstitium (Short arrow). (c) Testis of a healthy treatment rat (HT) with normal seminiferous tubules. And (d) Testis of a diabetic treatment rat (DT) with mild to moderate interstitial congestion (See arrow).

Similarly, the histological picture of the epididymis in DC rats revealed evidences of fragmentation and reduced density of intraluminal spermatozoa (Fig. 5b), while intraluminal

spermatozoa structure and population appear normal in HC, HT and DT rats (Fig. 5a, c and d respectively).

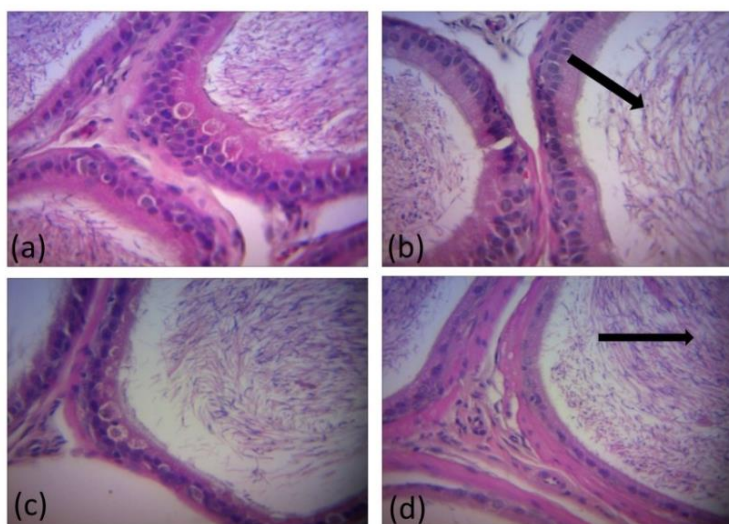


Fig. 5 Histopathological Photomicrographs (H & E, x400) showing; (a) Epididymis of a healthy control (HC) rat with no visible lesion. (b) Epididymis of a diabetic control (DC) rat with normal epithelium, but evidence of fragmentation and reduced density of intraluminal spermatozoa (See arrow). (c) Epididymis of a healthy treatment rat (HT) with no visible lesion. And (d) Epididymis of a diabetic treatment rat (DT) with improved spermatozoa population when compared to DC specimen (See arrow).

Discussion

Diabetes is a metabolic disorder of epidemic proportions and when left untreated the disease affects the organ systems of humans as well as animals in various ways. DM has been shown in previous studies to impact male reproductive fertility in addition to many other systemic effects (16). DM was induced in this study via a single intraperitoneal injection of STZ which resulted in marked elevation of FBS three days afterwards in the DC and DT animals at the onset of the experiment. The results of our study showed a deterioration in most of the reproductive parameters measured (sperm concentration, viability and normal morphology), as well as in testicular and epididymal histology in the untreated diabetic (DC) rats. Incidentally, rats in this group expressed abnormally high levels of testosterone and LH in their serum. This finding may appear to differ from the commonly reported trend of low serum testosterone in male diabetic subjects that exhibit poor reproductive parameters (17, 18). It is therefore important to note that the level of serum testosterone in male diabetic subjects is dependent on a number of factors.

One of such determinants of the level of serum testosterone is the type of diabetes mellitus induced. Low levels of serum testosterone is commonly found in T2DM, a picture described as hypogonadotropic hypogonadism, whereas, serum testosterone is usually significantly raised in T1DM especially in adolescents and middle aged men (19, 20). Another factor relevant to this finding is the pattern of change in body weight, since body mass index have been reported to relate inversely with serum testosterone concentration (20, 21). This is partly because testosterone is aromatized and converted to estradiol by aromatase enzyme in

adipocytes thereby reducing the concentration of testosterone in blood. In T1DM, tissue uptake of glucose (the primary source of energy) is compromised due to insulin deficiency caused by the destruction of insulin producing beta-islet cells of the pancreas (22). During conditions of insulin deprivation, there is marked metabolic alterations in the body that culminate in increased basal energy expenditure and profound protein catabolic state (23). This partly explains the weight loss commonly seen in T1DM and the high percentages of weight loss observed in DC and DT animals in this study. The catabolism of body fat mass implies less adipocyte will be available to convert testosterone to estradiol by aromatization, leading to a higher serum concentration of testosterone (24, 25). Significant loss of body fat may account for the severe weight loss observed in the diabetic control rats which suggest additional reason for the elevated serum testosterone in the animals.

The dosage and schedule of STZ administration in rats play a key role in determining the features of the DM induced. A single intraperitoneal injection of STZ at dosages between 40 – 60mg/kg is said to induce symptoms that mimic T1DM (26). This assertion is well corroborated in the results of this study with the finding of features like hyperglycaemia, severe weight loss and elevated serum testosterone and LH in the diabetic control group following single intraperitoneal injection of STZ at 55mg/kg. The concurrent elevation of both testosterone and LH suggests target organs' resistance to testosterone and failure of its negative feedback regulation of LH secretion from the anterior pituitary. DM has been previously reported to have adverse effects on the functionality of the hypothalamus-pituitary-gonadal axis, resulting in an abnormal sexual steroid feedback and this is

attributed to abnormal steroid transport and pituitary insensitivity (27). This may result as a consequence of the effect of diabetic autonomic neuropathy on the hypothalamus and the pituitary gland (18).

Furthermore, the sustained hyperglycemia ultimately leads to the generation of high levels of advanced glycation end-products (AGEs) which are injurious to spermatozoa and the process of spermatogenesis (11). The metabolic challenges induced by STZ in the diabetic animals will also contribute an increased production of reactive oxygen species (ROS) (17), which result in oxidative stress status when the level outnumber antioxidants, thereby culminating in deterioration of reproductive parameters (28).

The findings from this study clearly indicated that oral administration of the aqueous extract of *Basella alba* significantly reversed the observed deterioration in reproductive parameters (either partially or completely) in the diabetic treatment group when compared to diabetic control. This can be attributed to a number of beneficial effects of the extract that led to the reduction in percentage weight loss (reduced fat catabolism and increased testosterone aromatization) which might have contributed to lowering of serum testosterone back to within normal range in the DT group. Additionally, the restoration of normal testosterone and LH levels in DT rats may suggest that the plant extract is capable of stimulating enhanced target organ and pituitary sensitivity to testosterone, which restores proper negative feedback regulation along the hypothalamus-pituitary-testis axis. *Basella alba* has also been reported to contain numerous phytochemicals like flavonoids and phenolic compounds which are capable of neutralizing the oxidative potential of ROS by the donation of hydroxyl groups (29). The choice of

aqueous method of extraction in this study was informed by the fact that the aqueous extract is closest in composition to the form in which the plant is consumed after boiling in water. Additionally, unpublished data from our larger study suggested that the aqueous extract of *Basella alba* leaves possess better antioxidant effect when compared to a number of organic solvent extracts. The antihyperglycemic effect of the extract was also very evident in the result of this study, and this suggests a reduction in the generation of AGEs, thereby ameliorating the deleterious effect on the gonads and spermatozoa. However, the mechanism by which *Basella alba* exerts the antihyperglycemia cannot be explained within the scope of this study. Stimulation of pancreatic islet regeneration, up regulation of insulin receptors and possible insulin-like action by the plant extract itself are some of the possibilities that will be explored in future studies to unravel this observation.

Conclusion

The findings from this study confirmed a possible role for *Basella alba* in the management of male factor infertility secondary to T1DM. Regulation of the release and actions of reproductive hormones are important aspects of this beneficial effect of the plant. However, further studies involving an in-depth analysis of the phytochemical components and the in vivo antioxidant activities of the plant extract may be necessary to fully explain how this is achieved. Additionally, the administration of *Basella alba* leave extract for a longer duration and post-treatment cohabitation with female animals will give further information about the actual effect on fecundity of diabetic rats.

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