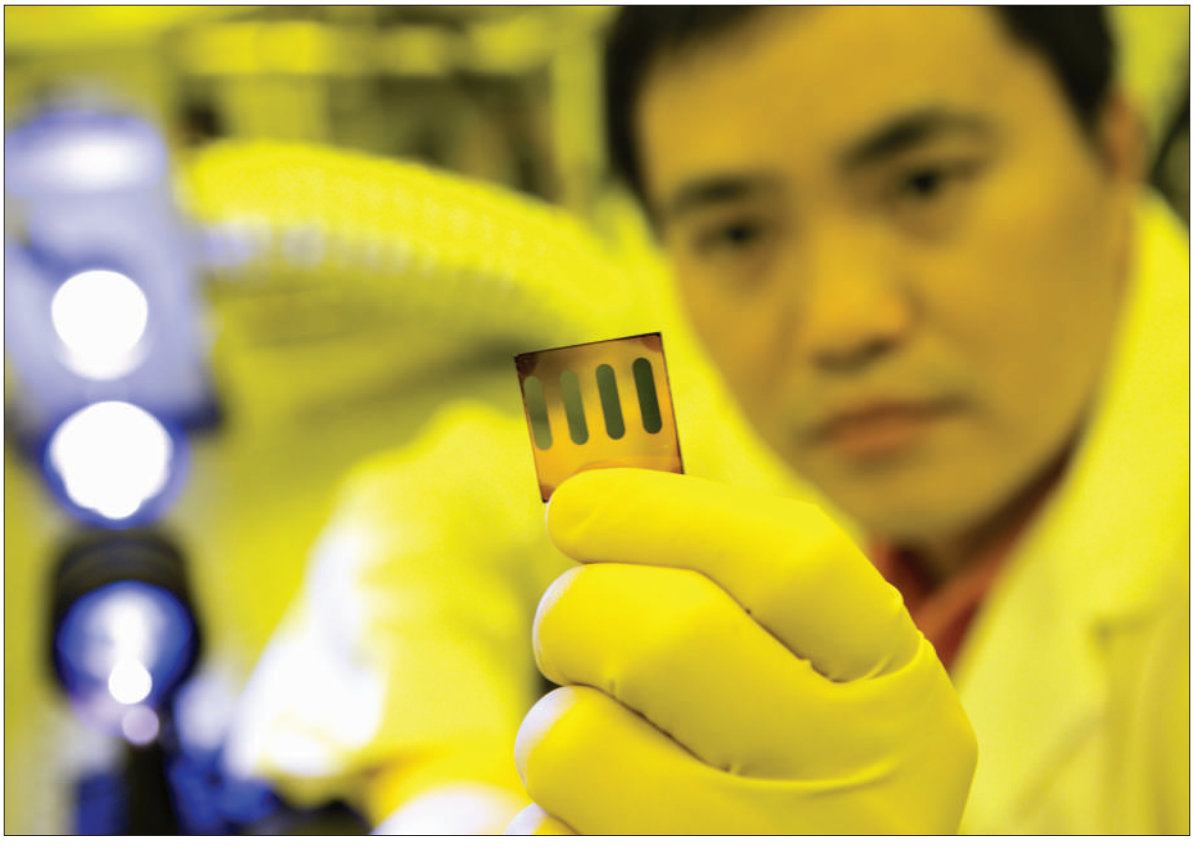

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Research Article
Histomorphometric Assessment of Seminiferous Tubules from Streptozotocin-nicotinamide-induced Diabetic Rats Treated with Unsweetened *Theobroma cacao* Powder

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Abstract

Background and Objective: Unsweetened cocoa powder (UCP) is antioxidant-rich and can mitigate oxidative stress-mediated testicular damage. The study assessed the impact of UCP on histomorphometry of seminiferous tubules (ST) in diabetic rats. **Material and Methods:** Twelve streptozotocin nicotinamide-induced hyperglycaemic rats aged 12-16 weeks were treated as follows: Diabetic cocoa (DC) received 400 mg/kg/day of 2% UCP drink (n = 6), diabetic untreated, DU (n = 6). Five normoglycaemic rats served as control (C). Fasting blood glucose (FBG) values were determined at baseline and biweekly. Six weeks later, rats were sacrificed. Blood was collected from the heart to determine plasma total antioxidant capacity (TAC) and testosterone levels. The right testes were processed and stained with periodic acid schiff (PAS). ST cross-sections and fertility status were determined using point counting and Johnsen's score respectively. **Results:** The mean baseline fasting blood glucose FBG of DU increased by 16.75% ($p>0.05$) whilst C and DC reduced by 17.26% ($p>0.05$) and 6.40% ($p>0.05$) respectively. The median cross-sectional area of STs in DC rats was significantly lesser than C's and significantly greater than DU's. Tubular germinal epithelium histology of DC was less damaged and scored a significantly higher median Johnsen's score for fertility status than DU. The mean serum testosterone level of DC was significantly lesser than DU and C. Mean plasma TAC of DC was significantly greater than DU but comparable to C. **Conclusion:** UCP mitigated testicular ST damage in diabetic rats by decreasing plasma glucose, maintaining high plasma total antioxidant capacity and preventing an increase in serum testosterone level.

Key words: *Theobroma cacao*, diabetes, streptozotocin, nicotinamide, stereology, point counting, seminiferous tubules, testosterone, total antioxidant capacity, Johnsen's score

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia resulting in microvascular and macrovascular complications as well as multi-organ damage involving the heart, kidney, brain and reproductive structures such as testis and ovary[1-3]. Diabetes mellitus increases one's risk of having impaired male reproductive functions[4,5]. This metabolic disorder is reported to impair spermatogenesis and reduces sperm count, sperm motility, seminal fluid volume and testosterone levels[6,7]. In diabetes, chronic hyperglycemia causes oxidative stress by generating free radicals in cells of the testis including those of spermatogenic series, Sertoli cells, Leydig (interstitial) cells and endothelial cells[8,9]. Free radicals can cause DNA damage and when not repaired, usually results in irreversible cell injury and subsequent necrosis and/or apoptosis[10]. Destruction of these vital cells especially those of spermatogenic series could bring about male infertility[11]. Due to the involvement of oxidative stress in chronic hyperglycemia-induced damage to reproductive structures as occur in diabetes, treatment or management with antioxidants stand a high chance of mitigating such damage[12,13].

Cocoa, also known as *Theobroma cacao*, is a rich antioxidant source even higher than red wine and tea[14,15]. Owing to cocoa's antioxidant potential, its regular administration has been useful in lessening deleterious effects on organs in oxidative-stress implicated experimental animal models such as chronic alcohol ingestion[16] and malaria[17]. In humans, cocoa has also demonstrated hypoglycaemic potential in oral glucose tolerance test (OGTT)[18-21]. In spite of these potentials, cocoa's histoprotective effect on reproductive structure such as the testis in diabetes mellitus has not been investigated. In addition, antioxidant levels of cocoa tend to decrease in more processed states such as sweetened chocolate or with the addition of milk[22]. Thus, less processed form such as unsweetened cocoa powder is being consumed as a beverage in order for one to harness its full protective benefits.

In the early phases of diabetes mellitus, histopathological changes are not very obvious but chronic hyperglycemia causes remodeling of tissues' morphometric variables such as number, length, cross-sectional area and volume[23-25]. Qualitative assessment of tissue pathology could be subjective and may indicate such tissues are normal even though they may have undergone morphometric changes that are part of the disease's pathophysiological process. In order to ascertain the extent of organ damage and/or progress of the disease with minimal human errors in both experimental and clinical diabetes mellitus, stereological techniques coupled with appropriate measurement tools could be used to empirically determine morphometric changes for the microscopic tissue profiles of interest[26,27]. Stereology involves the usage of unbiased random sampling methods to select slices of an organ for tissue processing, microtome sections for staining, as well as microscopic profiles on selected microtome sections. Application of the technique in the study of an organ using light and/or electron microscopy helps obtain a reproducible morphometric data which is representative of the organ[28]. Furthermore, the application could enhance understanding of mechanistic processes as occur in physiological or disease states by providing a fair assessment with minimal human errors[29]. Considering the additional advantage study of tissues using stereology-based measurements could present, the study sought to investigate the impact of unsweetened cocoa drink on histomorphometry of seminiferous tubule in testes of experimentally-induced diabetic rats.

MATERIALS AND METHODS

Study area: This study was conducted from February to July, 2018 in the Animal House of the School of Biological Sciences and laboratories of the Department of Biomedical Sciences and Department of Biochemistry, all in the University of Cape Coast, Ghana.

Drugs and chemicals: The materials and their source included Streptozotocin (Sigma-Aldrich Inc., St. Louis, Mo, USA), nicotinamide (Santa Cruz Biotechnology, Germany, sc-208096), metformin (Biomed Pharmacy, University of Cape Coast, Ghana), Glucoleader glucometer and glucose test strips and natural cocoa powder (Good Food Brand, Batch number AO1501A).

Diabetes induction, animal grouping and treatment administration: Twenty normoglycaemic (FBG$\leq$7.5 mmol L^{-1}) male Sprague-Dawley rats aged 12-16 weeks were procured from the Animal House of the University of Cape Coast. The rats were kept under ambient conditions of temperature 23-25°C, relative humidity (60±4%), 12 h light/dark cycle and were provided with a standard pellet diet (Grower Mash, Essaar, Ghana) and water *ad libitum*. Fifteen of them were fasted overnight and then injected sequentially with a single intraperitoneal injection of 60 mg kg^{-1} streptozotocin (STZ), dissolved in 0.1 M citrate buffer (pH 4.51) and then 20 min later, with 100 mg kg^{-1} nicotinamide (NCTD) dissolved in

physiological saline kept on ice. The injected rats were provided a 5% glucose solution overnight and diabetes was confirmed on seventh day post-intraperitoneal injection. Fasting blood glucose (FBG) levels of rats were estimated using GlucoLeader glucometer. STZ/NCTD injected rats with minimum fasting blood glucose of at least 7.6 mmol L^{-1} were confirmed diabetic[30]. Twelve of the injected rats became diabetic. A non-diabetic group of 5 rats was created and the rats were each injected with a dose-dependent volume of 60 mg kg^{-1} of citrate buffer and 100 mg kg^{-1} physiological saline kept on ice. After matching weight and blood glucose levels, the confirmed diabetic rats were selected into two groups of six, diabetic cocoa (DC) and diabetic untreated (DU). DC rats were provided daily with 400 mg kg^{-1} cocoa (2% v/v) via oral gavage while DU nor C rats received no cocoa drink. After the treatment, all the rats were provided with food and water *ad libitum*. The non-diabetic rats, control (C) were provided with water for 24 h. The treatment regimen lasted 6 weeks. FBG levels at baseline and end of the experiment were monitored biweekly after an overnight fast of 8 h. At the end of six-week treatment, the rats were anesthetized using chloroform inhalation, thoracotomy was performed and blood (V = 5 mL) was collected via cardiac puncture. The blood was centrifuged at 3000 rev min^{-1} and the serum was stored and later used for determination of plasma Total antioxidant capacity (TAC) and testosterone levels. The anesthetized rats were dissected and their testes harvested. The left and right testes were stored in 10% buffered formalin (pH 7.29) for 6 days.

Slicing, processing, sectioning and staining: The right testis was bisected longitudinally and the slices were processed using standard tissue processing protocol. Tissues were infiltrated with molten wax and embedded to form blocks for microtome sectioning. The wax-blocked tissues were initially trimmed using Leica microtome (Leica RM 2125, Germany) at a thickness of 10 µm to expose the whole profile of the tissue. The tissue was then sectioned at 5 µm. The first and tenth sections of the slice block were picked for periodic acid schiff (PAS) staining.

Stereological studies

Sampling and stereological study of seminiferous tubules: For each rat, five different fields of seminiferous tubules (ST) were randomly photographed at 4×objective using the Amscope camera connected to Olympus microscope. The cross-sectional areas of each seminiferous tubule in a section were determined as follows. Using Adobe Photoshop software, a grid of dimension 4 cm×4 cm was superimposed on each micrograph containing the seminiferous tubules (ST). Point counting was performed and the number of grid test points overlying profiles of seminiferous tubules within the counting frame was determined. The cross-sectional area (A) of each seminiferous tubule on each micrograph was computed using the equation:

$$A\ (ST) = P \times c^2$$

where, P is the number of grid test points overlying each seminiferous tubule and c is the actual value of the distance between two points on the test grid.

The actual magnification was determined by taking a photo of graduations on a 1 mm micrometer at 4×objective. The micrograph was uploaded and the software's grid was then superimposed on it. The magnification obtained was 1 cm:64 µm.

Assessment of fertility potential: Thirty photomicrographs of different fields of seminiferous tubules were taken from each slide. The germinal epithelium of the seminiferous tubules was graded on the scale 1-10 using Johnsen's score criteria described as follows[31]:

1 Neither germ cells nor sertoli cells present
2 No germ cells present
3 Only spermatogonia present
4 Only a few spermatocytes present
5 No spermatozoa or spermatids present but many spermatocytes present
6 Only a few spermatids present
7 No spermatozoa but many spermatids present
8 Only a few spermatozoa present
9 Many spermatozoa present but disorganized spermatogenesis
10 Complete spermatogenesis in testicular biopsy

The total Johnsen's score was obtained by dividing the total score by the number of tubules evaluated.

Total antioxidant capacity (TAC) of plasma: The total antioxidant capacity of the plasma was determined using the phosphomolybdenum method[32]. A reagent solution consisting of ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (0.6 M) was prepared by mixing the components in 1:1:1 ratio. Five hundred microlitres of the plasma were mixed with 3 mL

of the reagent solution in separate test tubes and the reaction mixture subsequently incubated at 95°C for 70 min. A blank solution was prepared by mixing 0.5 mL methanol and 3 mL reagent solution after cooling to room temperature, the absorbance of the test mixture was measured at 695 nm against the blank using spectrophotometer 500 µL of various concentrations (20-200 µg mL^{-1}) of the standard, ascorbic acid was each mixed with 3 mL reagent solution, incubated 95°C for 70 min and cooled. The absorbance was then determined against the blank solution after which the absorbance as ordinate was plotted against concentration as abscissa. The absorbance of the test plasma samples was interpolated on the standard line to generate the concentration of antioxidants, total antioxidant capacity (TAC) expressed in ascorbic acid (AscAE) equivalents.

Serum testosterone level: Serum testosterone level was determined using enzyme-linked immunosorbent assay (ELISA). Fifty microlitres (50 µL) of rabbit anti-testosterone reagent and 100 µL of testosterone-HRP conjugate, 10 µL of testosterone standard and 100 µL of serum were placed in goat anti-rabbit IgG-coated wells and incubated at 37°C for 90 min after which the microwells were rinsed with washing buffer. One hundred microlitres (100 µL) of TMB substrate was added and the reaction mixture mixed for 10 sec and subsequently incubated at room temperature (18-22°C) for 20 min. The reaction was stopped by addition of 100 µL stop solution. The absorbance of the reaction mixture was read at 450 nm with a microfilter well reader within 15 min.

Statistical analysis: Statistical analysis were performed using GraphPad Prism 7 statistical software. All data sets were tested for normal distribution using the Anderson-Darling test. Data sets with a p-value of less than 0.05 were concluded to be not normally distributed. Data on cross-sectional areas of seminiferous tubules did not follow normal distribution therefore median value was used as average. Kruskal-Wallis test was used to compare the median cross-sectional areas of seminiferous tubules among the experimental groups. Dunn's multiple comparison tests were then used to test for a significant difference in the variables between any two experimental groups. Parametric tests such as independent t-tests and one-way ANOVA were used to compare between or among experimental groups, data sets on FBG, serum testosterone level and plasma total antioxidant capacity (TAC). The $p<0.05$ was considered statistically significant.

RESULTS

Fasting blood glucose: The mean FBG (mmol L^{-1}) values of DU, DC and C at baseline and day 42 were 23.64±0.874 and 27.6±1.815 (16.75%, p = 0.0660), 24.68±3.430 and 23.10±0.379 (6.40%, p = 0.5340), 6.14±0.610 and 5.08±0.215 (-17.26%, p = 0.1397) respectively (Fig. 1).

Histological assessment of testis: Seminiferous tubules of diabetic untreated were reduced in cross-section such that many of the tubules appeared in cross-section as compared to the non-diabetic group (Fig. 2). When compared to the non-diabetic group, seminiferous tubules of the diabetic untreated group had a more thickened basement membrane and numerous pyknotic nuclei among cells of the seminiferous tubules as well as those of the tubulo-interstitium (Fig. 3). However, tubules of cocoa-treated diabetic groups were elongated and had bigger cross-sections (Fig. 2). The germinal epithelium of diabetic untreated appeared deranged unlike that of non-diabetic control and diabetic cocoa (Fig. 3).

Stereological (histomorphometry) assessment of seminiferous tubules: Median cross-sectional area ($\times 10^3$ μm^2) of the seminiferous tubules of non-diabetic (C), diabetic untreated (DU) and diabetic cocoa (DC) were 73.728, 16.384 and 202.752 respectively. The median cross-section of

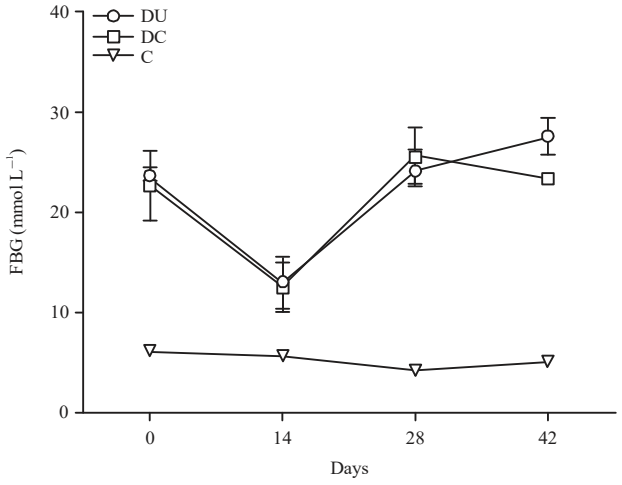

Fig. 1: Mean fasting blood glucose (mmol L^{-1}) for various experimental groups at baseline and end of experiment

DU: Diabetic untreated, DC: Diabetic cocoa, C: Non-diabetic, mean FBG of the baseline and day 42 for each experimental group were not significantly different from each other ($p \geq 0.05$)

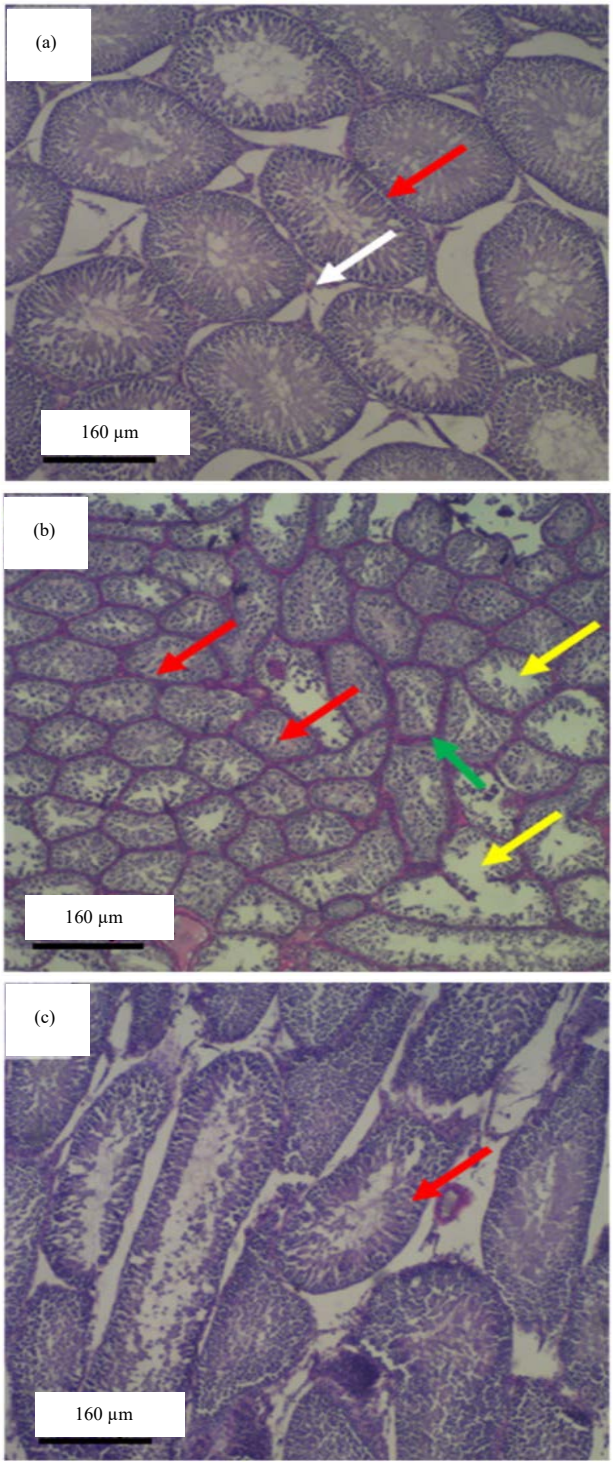

Fig. 2(a-c): Light micrograph of testis of experimental groups, (a) Non-diabetic, (b) Diabetic untreated and (c) Diabetic cocoa

Red arrows: Normal seminiferous tubules, Yellow arrows: Degenerated seminiferous tubules, Green arrow: Basement membrane, White arrow: Leydig cells

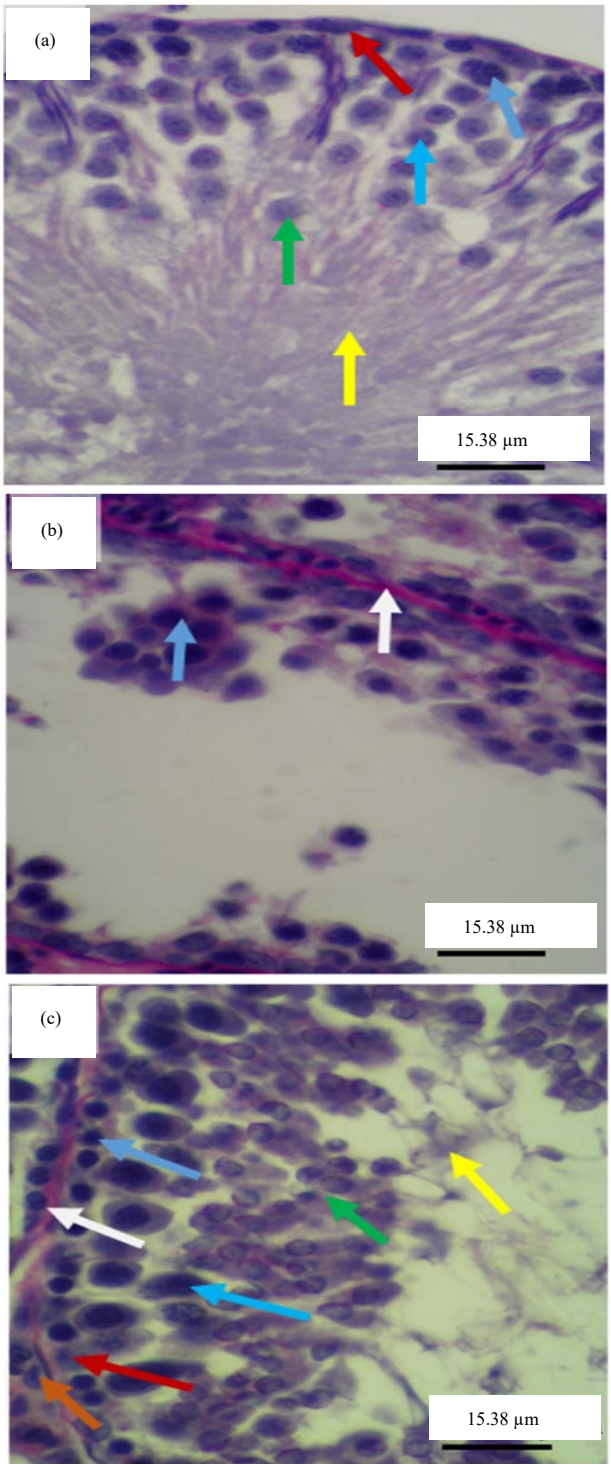

Fig. 3(a-c): Light micrograph of testis of experimental groups, (a) Non-diabetic, (b) Diabetic untreated and (c) Diabetic cocoa

Red arrows: Sertoli cells, Orange arrows: Spermatogonia, Blue arrows: Spermatocytes, Green arrows: Spermatids, Yellow arrows: Spermatozoa, White arrows: Basement membranes, Brown arrows: Leydig cells

Table 1: Median cross-sectional areas of seminiferous tubules of the various treatment groups

Treatment groups	MP	A ($\times 10^3$ µm^2)	K	p-value	CG (p-value)
Non-diabetic (C)	18.0^a	73.728	290.1	<0.0001	C: DU (<0.0001)*
Diabetic untreated (DU)	4.0^b	16.384			C: DC (0.0225)*
Diabetic cocoa (DC)	49.5^c	202.752			DU: DC (<0.0001)*

MP: Median point count for tubules, A: Median area of seminiferous tubules ($\times 10^3$ µm^2), K: Kruskal-Wallis statistic, CG: Comparison between groups, values which share different alphabets are statistically different, *p<0.05

Table 2: Median Johnsen's score of seminiferous tubules of the various treatment groups

Treatment groups	M	K	p-value	Mr	CG (p-value)	Mrd
Non-diabetic (C)	10	51.49	<0.0001	51.62	C: DU (<0.0001)	40.92****
Diabetic untreated (DU)	5			10.70	C: DC (0.0198)	15.49*
Diabetic cocoa (DC)	9			36.13	DU: DC (0.0001)	-25.43***

M: Median Johnsen's score, K: Kruskal-Wallis statistic, Mr: Mean rank, CG: Comparison between groups, Mrd: Mean rank difference, *p<0.05, **p<0.01,***p<0.001

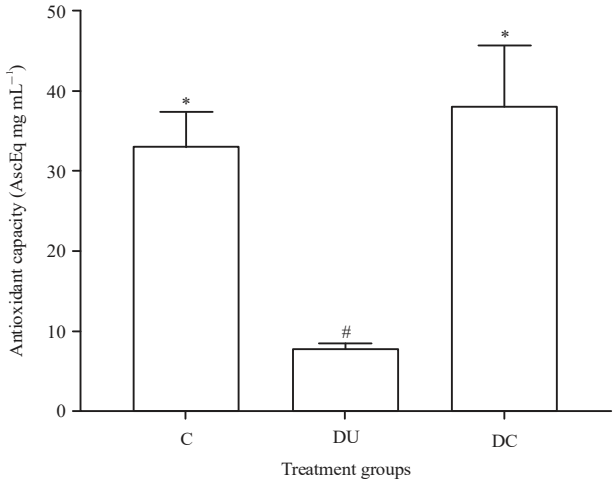

Fig. 4: Mean plasma total antioxidant capacity (TAC)/ AscEq (mg mL^{-1}) of experimental groups
DU: Diabetic untreated, DC: Diabetic treated with 400 mg kg^{-1} cocoa, C: Non-diabetic rats, groups which share different symbols are significantly different (p<0.05), mean plasma TAC of diabetic untreated group was significantly lesser than diabetic cocoa and non-diabetic control (p<0.05) whilst mean plasma TAC levels of non-diabetic and diabetic cocoa were not significantly different (p>0.05)

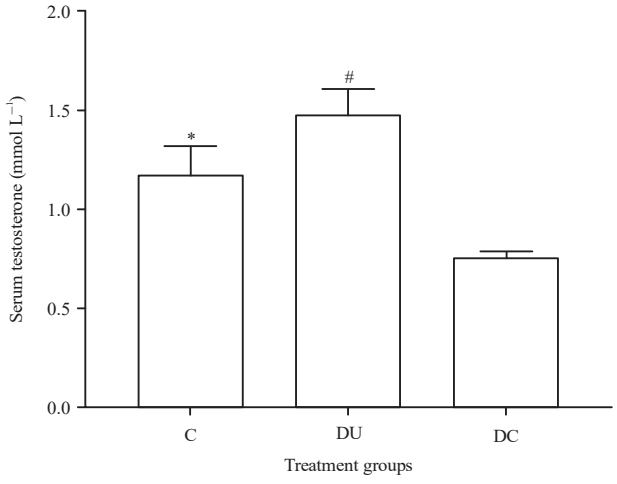

Fig. 5: Bar graph representing mean serum testosterone levels of various experimental groups
C: Non diabetic control, DU: Diabetic untreated, DC: Diabetic cocoa, one-way ANOVA test (F = 40.77) and *post hoc* Bonferroni's test showed that the mean serum testosterone levels of the various treatment group were significantly different from each other (p<0.05)

seminiferous tubules in group DU was significantly smaller than that of group C (p<0.0001). However, cross-sections of seminiferous tubules of DC were significantly greater than that of groups C (p = 0.0225) and DU respectively (p<0.0001) (Table 1).

Assessment of fertility potential using johnsen's score: Kruskal Walli's test showed that the median Johnsen's score among the various treatment groups was significantly different (p<0.05). Median Johnsen scores for non-diabetic, diabetic untreated and diabetic cocoa groups were 10, 5 and 9 respectively. The median score of the non-diabetic group was significantly greater than that of diabetic untreated and diabetic cocoa. The score of diabetic cocoa was significantly greater than that of diabetic untreated (Table 2).

Plasma total antioxidant capacity (TAC): The mean plasma TAC (AscEq µg mL^{-1}) of experimental groups C, DU and DC were 38.20±5.49, 7.90±0.52 and 33.1±2.48, respectively. Mean plasma TAC levels of DU were significantly lesser than non-diabetic and diabetic cocoa treatment groups (p<0.05). However, mean plasma TAC of the diabetic cocoa-treated group and non-diabetic groups were not significantly significant (p<0.05) (Fig. 4).

Serum testosterone level: The mean serum testosterone levels (nmol L^{-1}) of experimental groups C, DU and DC were 1.173±0.072, 1.473±0.066 and 0.740±0.022, respectively. Mean serum testosterone levels of DU were significantly higher than non-diabetic and diabetic cocoa treatment groups (p<0.05). The mean serum testosterone level of the non-diabetic group was significantly greater than that of the diabetic cocoa group (Fig. 5).

DISCUSSION

The purpose of the study was to investigate the effects of unsweetened cocoa on testicular histology in experimentally induced diabetic rats using the following criteria: a qualitative assessment of testicular histology, histomorphometric analysis of seminiferous tubules and prediction of fertility potential of the seminiferous tubules using Johnsen's score[31,33]. The fasting blood glucose, as well as antioxidant potential and testosterone level of the sera from the experimental groups, were also assessed.

Chronic hyperglycemia in diabetes is implicated in the pathophysiology of diabetic complications[34]. In most cells, chronic hyperglycemia causes increased intracellular glucose concentration resulting in activation of the increased polyol pathway, advanced glycation end-product (AGE) formation, activation of protein kinase C (PKC) isoforms and increased hexosamine pathway flux[35,36]. These culminate into oxidative stress thereby resulting in diabetes mellitus microvascular and macrovascular complications as it occurs in organs such as heart, lung, brain, kidney and testis. Therefore, reducing blood glucose and/or oxidative stress could delay chronic hyperglycemia-mediated testicular damage[13,33]. In this study, the cocoa-treated group recorded a 6.40% reduction of fasting blood glucose after 6 weeks of treatment unlike diabetic untreated which had a 16.75% rise (Fig. 1). This observation could be attributable to phytochemicals present in cocoa powder. Tannins (proanthocyanidins and ellagitannins) extracted from cocoa demonstrated *in vitro* inhibitory activities against alpha-amylase and glucoamylase[37] whilst another work has confirmed *in vitro* inhibitory activity of cocoa against both alpha-glucosidase and pancreatic amylase[38]. Ingestion of cocoa thus decreases postprandial glucose available for absorption into the bloodstream.

Oxidative stress has been implicated in cell nuclear damage and thus, a decrease in oxidative stress is paramount in salvaging the cells from dying[11,34,39]. One of the means of ascertaining the level of oxidative stress is to measure the total antioxidant capacity (TAC) of the plasma or serum[40,41]. TAC measures the concentration of resident antioxidants in tissue and is inversely proportional to the level of oxidative radicals. Though not specific as compared to other antioxidant assays such as superoxide dismutase (SOD) or glutathione (GSH), TAC can, however, predict the level of oxidative stress in a tissue. In this study, serum TAC level was determined and the diabetic untreated group had a significant lower TAC level than the non-diabetic group. On the other hand, the cocoa-treated diabetic group had a significantly higher plasma total antioxidant capacity than diabetic untreated and higher level, though not significant, to the non-diabetic group (Fig. 4). It has been reported that continuous ingestion of cocoa increases plasma antioxidant levels. Cocoa powder contains antioxidants predominantly, epicatechins and catechins[42]. Model organisms used to investigate the effect of cocoa polyphenols on resistance to oxidative stress showed that polyphenol-rich cocoa powder resulted in increased resistance to hydrogen peroxide tolerance[43]. Antioxidants are useful in biological systems as they rid the cell of oxidative radicals which causes nuclear damage and cell death[34]. Oxidative stress in cells is reported to inhibit cellular glucose utilization via the glycolytic pathway and Kreb's cycle and indirectly causing an increase in plasma glucose[36]. The cocoa treated diabetic group recorded a decrease in mean plasma glucose but higher plasma TAC level whilst the diabetic untreated group recorded an increase in blood glucose but a lower plasma TAC level. Thus, cocoa could also help the utilization of glucose via the glycolytic pathway.

Experimental and clinical diabetes cause histological changes that decrease an organ's optimal functioning. Histological sections of the testis revealed that the diabetic untreated group unlike the non-diabetic had shrunken seminiferous tubules, thickened basement membrane and severely disrupted germinal epithelium in the seminiferous tubules (Fig. 2). Numerous pyknotic nuclei were observed in all strata of the germinal epithelium in the seminiferous tubules of the diabetic untreated group when compared with the non-diabetic group which had no such changes (Fig. 3). Exposure of seminiferous tubules to chronic hyperglycemia is associated with progressive testicular disruption[33]. Chronic hyperglycemia causes cytoplasmic buildup of free oxygen radicals which damage deoxyribonucleic acid (DNA) in the nucleus resulting in pyknosis, clumping of nuclear material[10]. The cocoa treated diabetic group unlike the diabetic untreated group had lesser pyknotic changes in the germinal epithelium. These could be attributed to the antihyperglycaemic and/or antioxidant potential of cocoa powder. The stereological assessment conducted on testicular tissue in this study showed that the diabetic untreated group had a significantly lesser median cross-sectional area of seminiferous tubules when compared to the non-diabetic group (Table 1). Interestingly, the cocoa treated diabetic group had no shrunken tubules but mostly elongated forms denoting the effect of cocoa. The diameter of seminiferous tubules is reported to have a correlation with serum testosterone levels. Testosterone, a steroid hormone is necessary for the normal development of the male reproductive system. The hormone is converted to its biologically active form dihydrotestosterone by the enzyme

5α-reductase[44]. However, hypertestosteronemia is reported to be detrimental to the organs of the male reproductive system and other organs such as kidney[45-48] and liver[47,49]. Hypertestosteronemia could result in shrinkage of the seminiferous tubules[50]. The diabetic untreated group had a higher mean serum testosterone level than the non-diabetic group which may explain the shrunken tubules (Fig. 5). However, this finding is contrary to the widely reported low serum testosterone levels among diabetics in both experimental and clinical diabetes[51]. The high serum testosterone could be as a result of defective feedback mechanism. Leydig cells present in the testicular tubulointerstitium synthesize and secrete testosterone under the influence of luteinizing hormone (LH)[52]. Sertoli cells which are present in the seminiferous tubule secrete inhibin to inhibit pituitary secretion of the gonadotropin as a response to high testosterone levels[52]. With severe destruction of the cells in the seminiferous tubules, including sertoli cells, negative feedback control on the release of luteinizing hormone is likely to be defective thereby resulting in high testosterone levels. The cocoa treated diabetic group had a significantly lower serum testosterone level than the non-diabetic group. The histological assessment confirmed the preservation of more seminiferous tubule cells unlike diabetic untreated.

Infertility is widely reported among diabetic males owing to defective spermatogenesis resulting in oligozoospermia or azoospermia[6,53]. As a follow up to the disruption in the germinal epithelium, the fertility potential of the tubules was assessed using the Johnsen's score system. The diabetic untreated had a significantly lesser Johnsen's score than the non-diabetic group. The cocoa treated group had a lower Johnsen's score than the non-diabetic group however, the cocoa-treated group had a better and higher median Johnsen's score than the diabetic untreated group (Table 2). On average, seminiferous tubules of the diabetic untreated group had no spermatozoa or spermatids present but many spermatocytes present whilst the cocoa treated group had many spermatozoa present but disorganized spermatogenesis and non-diabetic group, complete spermatogenesis. Thus, the diabetic untreated group may record low or no sperm count putting them at risk of incapable of producing viable sperms for fertilization of the secondary oocyte. Cocoa, therefore, minimized damage to cells of the spermatogenic series. Aside from antioxidants present in cocoa, it also has a high amount of zinc[54] which guards sperms against bacteria and, protects sperm from free radicals, compounds that damage cells due to its antioxidant properties[55]. Zinc has the ability to improve the number and healthiness of sperm and thus, its presence was able to improve upon spermatogenesis. Sperm count and serum estrogen levels were however not assessed in this study.

CONCLUSION

Cocoa minimized shrinkage of seminiferous tubules and derangement of the germinal epithelium of seminiferous tubules. These protective abilities of cocoa could be attributable to phytochemicals, mainly the polyphenols and minerals which exert antihyperglycaemic and antioxidative potentials. Hence, regular consumption of unsweetened powder of *Theobroma cacao* improved testicular health and therefore could be used in improving fertility potential in diabetic males.

SIGNIFICANCE STATEMENT

This study discovered that regular intake of unsweetened cocoa powder (UCP) protects seminiferous tubules from atresia and tubular germinal epithelium from damage in hyperglycaemic rats. UCP could be beneficial in increasing testicular fertility potential in diabetic men. This study could not explore the effect of UCP on sperm count and quality, specific cells in the spermatogenic series, specific testicular antioxidant system and anti-inflammatory potential.

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