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Comparison of streptozotocin-induced diabetic and insulin resistant effects on spermatogenesis with proliferating cell nuclear antigen (PCNA) immunostaining of adult rat testis

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ABSTRACT

Male reproductive function is one of the mammalian systems that are impaired by diabetes mellitus and on the average, it will affect many more men prior to and during their reproductive years. The present study aimed to compare proliferating cell nuclear antigen (PCNA) immunostaining effects at stage VII of the spermatogenic cycle in streptozotocin-induced and insulin resistant diabetic rat testis. Male adult Sprague-Dawley rats (120-140 g) were randomly divided into 3 groups. Group 1: control group; fed on normal rat pellets. Group 2: streptozotocin diabetic group; received a single dose IP injection of streptozotocin 45 mg/kg BW in Na^+ citrate buffer pH 4.5. Group 3: insulin resistant diabetic group; fed ad libitum on a special diet containing 25% fructose W/W. Following hyperglycaemia confirmation, animals were perfused with 4% Paraformaldehyde (PFA). Testes were isolated, and fixed in 4% PFA overnight, embedded in paraffin, 5µm thick sections were made and mounted on poly-L-lysine coated slides. Immunohistochemical staining was carried out on the positively charged slides using the PCNA as primary antibody.The results of this study showed that diabetes mellitus induced by streptozotocin injection and chronic fructose consumption through insulin resistance significantly (P<0.05) reduced PCNA index, mean seminiferous tubular diameter and testicular diameter. It could also be inferred from the results that diabetes mellitus and insulin resistance may necessarily not impair differentiation of primordial germ cells (gonocytes) into Type A and Type B spermatogonia.
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1. Introduction

Diabetes mellitus is a common health problem with many functional and structural complications (Cade, 2008). The World Health Organization (WHO) in year 2000 reported that 177 million people were affected by diabetes worldwide and projected that by 2025, this figure will rise to over 300 million (WHO, 2002). Likewise (Zimmet et al., 2001) also projected that this figure will be doubled in the next 20 years.

According to the American Diabetic Association, diabetes is characterized by hyperglycaemia, which often results from defects in insulin secretion, insulin action, or both (American Diabetic Association, 2011). Rakietan et al. (1963) first demonstrated the diabetogenic property of Streptozotocin (STZ) in dogs and rats. This was followed by the works of Junod et al. (1967) who stated that STZ causes hyperglycaemia mainly by its direct cytotoxic action on the pancreatic beta cells. More recently, Ozturk et al. (1996) while comparing STZ and Alloxan, stated that STZ is a preferred agent to induce Type I experimental diabetes since it has some advantages over Alloxan. Some of these advantages are longer half-life, sustained hyperglycaemia and the development of well characterized diabetic complications with fewer incidences of ketosis as well as mortality.

Fructose is a known hexose like glucose but differs by the presence of a keto group in C2 compared to the aldehyde group in C1 of glucose (Luc and Kim-anne, 2010). Likewise, its metabolism is different from that of glucose and sucrose, and this has been reported to contribute to its efficacy to induce type 2 diabetes mellitus via insulin resistance (decrease insulin action) in rats and in genetically predisposed humans. For example, it's been reported that fructose does not require insulin for its transport into hepatic cells or initial step of its

hepatic metabolism (Luc and Kim-anne, 2010). Fructose also increases fasting glycaemia (Liu et al., 2006) and has been reported to cause hepatic insulin resistance in healthy men (Faeh et al., 2005). Yi and Zhongjie (2010) stated that insulin resistance (IR) refers to suppressed or delayed responses to insulin and it's generally a 'post-receptor' effectwith the cells being unresponsive to insulin rather than a problem with insulin production.

The testis is a highly proliferative tissue with sperm cells (spermatozoa) produced from stem cells (spermatogonia) by two consecutive steps of cell multiplication and spermatid cytodifferentiation. Mitotic proliferation of spermatogonia generates primary spermatocytes which enter meiosis, leading to the generation of spermatids. Stephan et al. (1996) stated that the number of cells entering meiosis is constant because the spermatogonia or premeiotic spermatocytes are eliminated by apoptosis.

PCNA is a 36kDa non-histone protein found in the nucleus that plays a role in the initiation of cell proliferation by mediating DNA polymerase, which plays an integral role in the eukaryotic cell cycle and is essential for cellular DNA synthesis. PCNA is a primary antibody; its levels are elevated in the S, G2, and M phases of cell mitosis in normal and malignant tissues. Its biological half-life is 20 hours and its expression has a broad correlation with mitotic activity and can be used as a marker for cell proliferation (Bravo and Macdonald-Bravo, 1987; Waseem and Lane, 1990; van Dierendonck et al., 1991; Dervan et al., 1992; Linden et al., 1992). PCNA is useful for the diagnosis of germinal arrest because there are significantly reduced PCNA levels in germinal arrest, which is an indication of DNA synthesis deterioration (Zeng et al., 2001).

Diabetes is also caused by complicated factors e.g. absolute insulin deficiency and relative insulin deficiency i.e. insulin resistance and its damage to the male reproductive system has been reported to threaten men's health (Zhao et al., 2004). Male sexual and reproductive functions are one of the mammalian systems that are clearly impaired by Diabetes (Guneli et al., 2008; Jelodar et al., 2010). In line with this view, Sexton and Jarow, (1997) stated that diabetes mellitus will affect many more men prior to and during their reproductive years (Sexton and Jarow, 1997).

These authors also reported that diabetes affects male reproductive function at multiple levels due to its effects on the endocrine control of spermatogenesis, spermatogenesis itself, impairing penile erection and ejaculation (Sexton and Jarow, 1997). Likewise, Arikawe et al. (2006) reported that diabetes mellitus and insulin resistance affect semen parameters and impair spermatogenesis in male rats. Thus, the present study aimed to compare PCNA immunostaining effects at stage VII of the spermatogenic cycle in streptozotocin-induced and insulin resistant diabetic rat testis.

2. Materials and methods

Twenty-one adult male Sprague-Dawley rats, whose average weight ranged between 120-140g were procured from a breeding stock maintained in the Laboratory Animal Department of the College of Medicine, University of Lagos. The animals were housed in clear polypropylene cages lined with wood chip beddings and were allowed to acclimatize in the Physiology Department animal laboratory with an ambient temperature maintained between 26ºC-28ºC for a period of one week before the beginning of the study. The rats were also maintained under standard colony photoperiodic conditions with a 12-hour light/12-hour dark cycle (lights on at 7:00 hour) and all animals had unrestricted (ad libitum) access to water. The rats were randomly divided into 3 groups (of 7 animals each of similar weight).

Group 1 served as the Control group, fed on normal rat chow throughout experimental period of 12 weeks. Group 2 served as Streptozotocin diabetic group; fed on normal rat chow and received a single dose IP (intraperitoneal) injection of Streptozotocin, 45 mg/kg body weight (Guneli et al., 2008) freshly dissolved in Na^+ citrate buffer pH 4.5 for 4 weeks. Blood samples were collected from the tail vein 48 hours after Streptozotocin injection to confirm hyperglycaemia using Dextrostix Test Strips (Bayer Corporation, U.K.) following the glucose oxidase method (Hugget and Nixon, 1957). Group 3 served as insulin resistant diabetic group; fed ad libitum on a special diet containing 25% fructose mixed with 75% normal rat chow (w/w) for 4 weeks (Arikawe and Olatunji-Bello, 2004) and continued till the 12th week (Arikawe et al., 2006). Hyperglycaemia was confirmed at the 12th week using Dextrostix Test Strips (Bayer Corporation, U. K.) following the glucose oxidase method (Hugget and Nixon, 1957). Polydipsia, polyuria and polyphagia were observed (Jelodar et al., 2010) and confirmed in the experimental groups (groups 2 and 3). All animals had free access to drinking water throughout the duration of the study. Rats with blood glucose concentration above 250 mg/dl were used as Type 1 diabetic rats (Akingba and Burnett, 2001), while rats with blood glucose concentration above 200 mg/dl were used as insulin resistant diabetic rats (Catena et al., 2003). Rats were weighed weekly throughout the duration of the experiment and animals were monitored for general health during the treatment period. All the procedures were performed in accordance with the guidelines of the College Ethical Committee on the use of laboratory animals for research.

Methodology

At the end of each experimental period, following hyperglycaemia confirmation, the following analyzes were carried out (i) Haematologic analysis for Glycosylated Haemoglobin levels (ii) Immunohistochemical staining using PCNA as primary antibody.

Immunohistochemical staining

At the end of each experimental period, following hyperglycaemia confirmation, animals were perfused using standard perfusion techniques in the laboratory with 4% paraformaldehyde (PFA), this was carried out at high stringency. Summarily before removal of the testes, the rats were deeply anaethetized with diethly ether and pain suppressed using sodium pentobarbitone. Rats were then perfused transcardially first with Dulbecco phosphate buffer saline (PBS) and followed by 4% PFA in PBS. Successful perfusion was confirmed by spontaneous movement (formalin dance), rapid tail rise, stretching, lightened colour of liver and pale testis. Testes were isolated, weighed and fixed in 4% PFA overnight; they were passed through ascending series of ethanol baths, embedded in paraffin, 5μm thick sections of paraffin-embedded samples were made using the microtome machine and mo-

unted on poly-L-lysine coated slides i.e. positively charged slides. Routine deparafinization and rehydration steps were carried out, followed by antigen retrieval steps (ARs). Antigen retrieval (or antigen recovery) is performed to expose or retrieve antigens which have become masked by the tissue fixation process.

Following successful antigen retrieval steps, immunohistochemical staining was carried out on the positively charged slides using the PCNA as primary antibody. The slides were incubated in 1% bovine serum albumin in PBS (blocking solution) for 30 minutes at room temperature and washed 3 times in PBS; slides were incubated in pre-diluted primary antibody (PCNA, Invitrogen USA) for 60 minutes at room temperature in a humidified chamber and then washed with PBS twice for 5 minutes each.

Further immunostaining was performed using labeled Streptavidin Horse Radish Peroxidase (HRP) for another 60 minutes at room temperature in a humidified chamber and then washed with PBS twice for 5 minutes each; final step of antibody localization of peroxidase deposition was achieved by using Diaminobenzidine-H_2O_2 (DAB, Dako) for chromogenic reaction and subsequently washed in running water for another 5 minutes; this was followed by counterstaining with Harris' haematoxylin and eosin, dehydrated in alcohols to xylene and finally mounted using dibutylpthalate xylene (DPX) with coverslip. As a control for method specificity, in negative control slide, the step using primary antibody (PCNA) was omitted and slides were viewed under the light microscope.

Ten seminiferous tubules were counted in each slide; cells with brown nuclear staining were considered positive (Altay et al., 2003). Both stained and non-stained germ cells were counted and the ratio of stained cells to the total number germ cells, i.e. PCNA index and density was calculated (Altay et al., 2003). The average PCNA index in each case was obtained by dividing the sum of all PCNA indices by the number of seminiferous tubules in which the calculation was carried out. Testicular diameter (TD) was measured directly by cutting testes vertically into 2 and largest TD then measured (Altay et al., 2003). Mean seminiferous tubule diameter (MSTD) was evaluated by measuring the smallest diameter of 10 tubuli on 10 randomly selected fields using an ocular micrometer at x400 magnification (Altay et al., 2003).

Statistical analysis

All data are presented as mean±standard error of mean (SEM). The data was analyzed using One-way ANOVA (analysis of variance) followed by Student-Newman-Keuls post-hoc test. Level of statistical significance was taken at P<0.05. The image J software package was used to analyze the immunohistochemical slides.

3. Results

Fasting blood glucose concentration (mg/dl) and glycosylated haemoglobin level (%) in the control rats was (91.4±2.1mg/dl, 5.5±0.2%); the streptozotocin diabetic rats (520.5±34.2mg/dl, 9.6±0.1%); and the insulin resistant diabetic rats (203.2±1.3mg/dl, 8.9±0.1%) respectively. These were significantly lower (P<0.001) in the control rats compared to rats in the experimental groups (Table 1).

MSTD (µm) of the left testis in the control rats was (201.5±6.4 µm); the streptozotocin diabetic rats (192.0±16.1 µm); and the insulin resistant diabetic rats (155.8±7.0 µm). Thus, MSTD of the left testis was significantly lower (P<0.05) in insulin resistant diabetic rats compared to the Control group while there was no significant difference between the control rats and streptozotocin diabetic rats. On the other hand, MSTD of the right testis was significantly lower (P<0.001) in the streptozotocin diabetic rats (177.8±6.6 µm); the insulin resistant diabetic rats (150.5±10.7 µm); compared to rats in the control (219.8±9.4 µm) group. Likewise, MSTD of both testes was significantly lower (P<0.001) in the streptozotocin diabetic rats (189.4±8.8 µm); and insulin resistant diabetic rats (153.2±6.2 µm) compared to rats in the control (210.6±6.1 µm) group. Testicular diameter (mm) in the control rats was (15.7±0.4 mm); streptozotocin diabetic rats (11.8±0.8 mm); insulin resistance diabetic rats (9.5±0.2 mm). Testicular diameter was significantly lower (P<0.001) in both experimental groups compared to rats in the control group (Table 1).

Table 1. Fasting blood glucose concentration, glycosylated haemoglobin level, MSTD, and TD in control, streptozotocin diabetic and Insulin resistant diabetic groups

	Control	Streptozotocin diabetic	Insulin resistant diabetic
(FBG) (mg/dl)	91.4±2.1	520.5±34.2£*	203.2±1.3£
HbA1c (%)	5.5±0.2	9.6±0.1£*	8.9±0.1£
MSTD (µm) Left Testis	201. 5±6.4	192.0±16.1	155.8±7.0α
MSTD (µm) Right Testis	219.8±9.4	177.8±6.6£	150.5±10.7£
MSTD (µm) Both Testes	210.6±6.1	189.4±8.8α*	153.2±6.2£
TD (mm)	15.7±0.4	11.8±0.8£*	9.5±0.2£

All results presented in mean±SEM
£P<0.001 Vs Control; αP<0.05 Vs Control; *P<0.001 Vs Insulin resistant diabetic
Footnote: Mean Seminiferous Tubule Diameter (MSTD); Testicular Diameter (TD); Glycosylated Haemoglobin level (HbA1c (%); Fasting Blood Glucose (FBG)

PCNA index (%) in the left testis was significantly lower (P<0.001) in streptozotocin diabetic rats (63.2±0.1%); and insulin resistant diabetic rats (21.3±0.1%) compared to the rats in the control group (82.8±0.1%). In the right testis, PCNA was also significantly lower (P<0.001) in the streptozotocin diabetic rats (69.0±0.1%); and insulin resistant diabetic rats (47.4±0.2%) compared to the rats in the control group (88.4±0.1%). Likewise, PCNA index in both testes was significantly lower (P < 0.001) in the streptozotocin diabetic rats (66.1±1.3 %); and insulin resistant diabetic rats (34.4±5.7%) compared to the rats in the control group (85.5±1.2%) (Table 2).

Numerical density (ND) of seminiferous tubule (µm-2) of the left testis in control rats was (0.04±0.005 µm-2); streptozotocin diabetic rats (0.04±0.002 µm-2); and insulin resistant diabetic rats (0.02±0.003 µm-2). Thus, ND of the left testis was significantly lower (P<0.05) in insulin resistant diabetic rats compared to the control group while there was no significant difference between the control rats and streptozotocin diabetic rats. On the other hand, ND of the right testis was significantly lower (P<0.001) in the streptozotocin diabetic rats (0.08±0.005 µm-2); and insulin resistant diabetic rats (0.02±0.002 µm-2) compared to the control rats (0.2±0.02 µm-2). Likewise, ND of both testes was

Table 2. PCNA index and numerical density in control, streptozotocin diabetic and insulin resistant diabetic groups

	Control	Streptozotocin diabetic	Insulin resistant diabetic
PCNA Index (%) Left Testis	82.8±0.1	63.2±0.1£*	21.3±0.1£
PCNA Index (%) Right Testis	88.4±0.1	69.0±0.1£*	47.4±0.2£
PCNA Index (%) Both Testes	85.5±1.2	66.1±1.3£*	34.4±5.7£
Numerical Density (µm-2) Left Testis	0.04±0.005	0.04±0.002	0.02±0.003£
Numerical Density (µm-2) Right Testis	0.2±0.02	0.08±0.005£*	0.02±0.002£
Numerical Density (µm-2) Both Testes	0.14±0.05	0.06±0.01£	0.02±0.003£

All results presented in mean±SEM
£P<0.001 Vs Control; αP<0.05 Vs Control; *P < 0.001 Vs Insulin resistant diabetic

significantly lower (P<0.001) in the streptozotocin diabetic rats (0.06±0.001µm-2); and insulin resistant diabetic rats (0.02±0.003 µm-2) compared to the control rats (0.14±0.05 µm-2). (Table 2). The immunohistochemical staining of seminiferous tubules in the three groups showed that positive slides for all the groups were reactive i.e. nuclei of the cells were brown coloured stained. Thus are positive for PCNA staining. In Figure 1, all the cell lines were present and no abnormality was detected. In Figure 2, secondary spermatocytes and elongated spermatids were not identified.

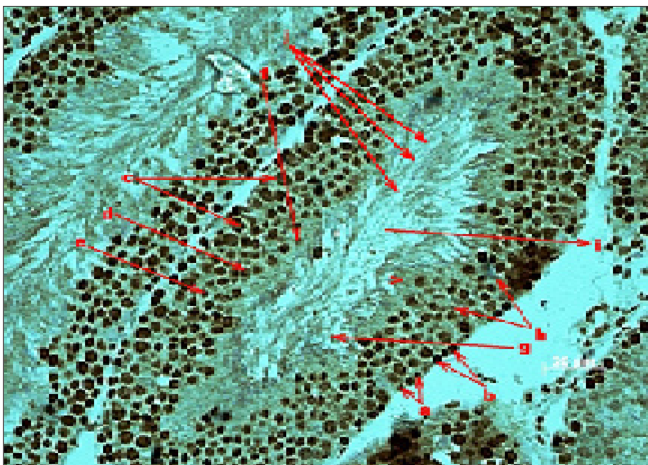

Fig. 1. Spermatogenic cycle in control seminiferous tubule x200
a- Type A spermatogonia
b- Type B spermatogonia
c- Primary spermatocytes
d- Secondary spermatocytes
e- Round spermatid
f- Older spermatid
g- Elongated spermatid
h- Sertoli cells
i- Seminiferous lumen
j- Spermatozoa

4. Discussion

Streptozotocin (STZ) and alloxan (ALX) are two chemicals used to induce experimental type I diabetes mellitus in adult animals. Streptozotocin was chosen over alloxan for use in this study in line with the view of Ozturk et al. (1996) who

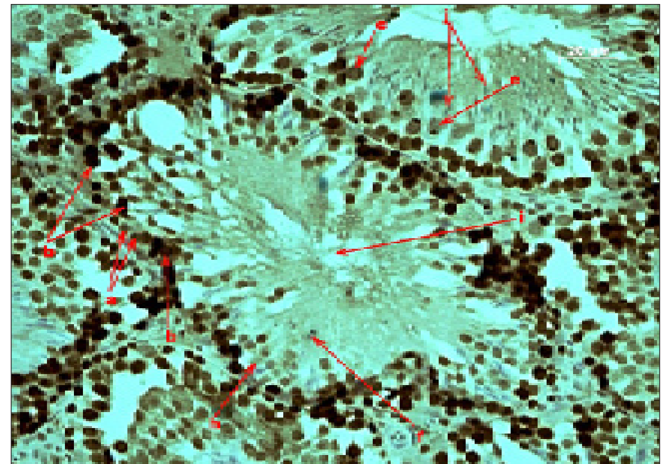

Fig. 2. Spermatogenic cycle in streptozotoc in diabete seminiferous tubule x200
a- Type A spermatogonia
b- Type B spermatogonia
c- Primary spermatocytos
d- 2º Spermatocytes not identitied
e- Round spermatid
f- Older spermatid
g- Elongated spermatids not identited
h- Sertoli cell
i- Siminiferous lumen
j- Spermatozoa

reported that it has some advantages over alloxan like sustained duration of hyperglycaemia and the development of well characterized diabetic complications with fewer incidences of ketosis as well as mortality (Ozturk et al., 1996). Also streptozotocin has been reported to have greater stability and relatively lacks extrapancreatic toxicity when compared to alloxan (Ioannides, 1996). The last point is very important because the present study is on the male reproductive function (spermatogenesis).

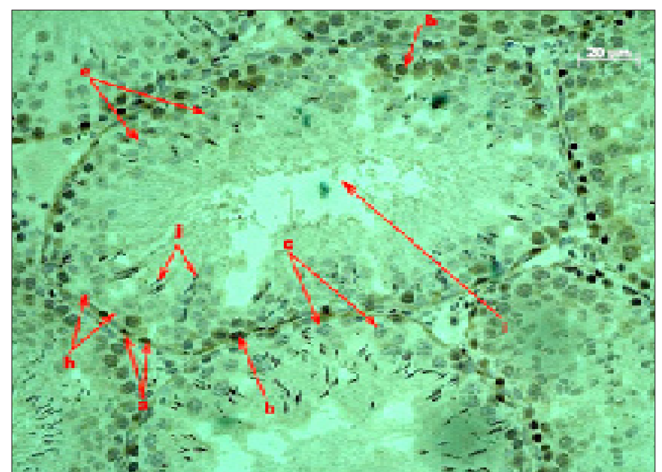

Fig. 3. Spermatogenic cycle in insülin resistant seminiferous tubule x200
a- Type A spermatogonia
b- Type B spermatogonia
c- Primary spermatocyles
d- 2º Spermatocytes not identified
e- Round spermatid
f- Older spermatids not identified
g- Elongated spermatids not identified
h- Sertoli cell
i- Seminiferous lumen
j- Spermatozoa

Fasting blood glucose (FBG) and glycosylated haemoglobin (HbA1c) were significantly higher (P<0.001) in both experimental groups compared to rats in the control group. The results on FBG level support the views that streptozotocin increases blood glucose in rats (Altay et al., 2003;Gunelli et al., 2008; Lee et al., 2010; Fernandes et al., 2011) to cause type I diabetes mellitus and that chronic fructose consumption through insulin resistance mechanism causes type II diabetes mellitus (Arikawe et al., 2006; Arikawe et al., 2011).

The glycaemic level was well controlled in the control animals (HbA1c<6.5%) while in the streptozotocin diabetic and insulin resistant diabetic groups, it was not well controlled (HbA1c>6.5%) (Bonnefont-Rousselot et al., 2000). This is also in line with the view of Punithavathi et al. (2011).

PCNA reacts intensely with the nuclei of cells at the preleptotene, leptotene through pachytene stages of spermatogenesis but it does not react with the nuclei of Sertoli cells (Wrobel et al., 1996). This was observed in the positive slides (with primary antibody) for all the groups which were reactive i.e. nuclei of the cells were brown colour stained. Thus, were termed positive for PCNA (Altay et al., 2003). Furthermore, there was necrosis (focal or global) and reduction (mild or moderate) in germ (viable) cells and spermatids in both treated groups compared to the control group.

MSTD of both testes was significantly lower (P<0.001) in the experimental groups compared to the Control group. This effect was slightly more on the right testis than the left testis and progressed with the duration of the diabetic state. TD, PCNA index (%), and ND were also significantly reduced (P <0.001) in all the rats in the experimental groups compared to the rats in the control group. This is in line with the view of Altay et al. (2003) and Jelodar et al. (2010). This confirms that diabetes mellitus and insulin resistance impair spermatogenesis in male rats (Arikawe et al., 2006) and could also alter conventional semen parameters like semen volume, sperm count, motility and morphology in humans (La Vignera et al., 2011). The results on MSTD, PCNA and ND can be used as morphological indicators of spermatogenesis failure (Cameron et al., 1985; Cai et al., 2000) especially since the spermatogenic cell cycle series were decreased in the streptozotocin diabetic and insulin resistant diabetic groups.

The results of this study showed that diabetes mellitus and insulin resistance reduced PCNA index, mean seminiferous tubular diameter and testicular diameter. It could also be inferred from the results that diabetes mellitus and insulin resistance may necessarily not impair differentiation of primordial germ cells (gonocytes) into Type A and Type B spermatogonia.

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