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Effects of Diabetes Mellitus on the Reproductive System of Adult Male Mice After One Cycle of Spermatogenesis

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Abstract

Background: Diabetes mellitus is a disease characterized by chronic hyperglycemia. Poor management of hyperglycemia leads to complications that can reduce quality of life. Diabetes mellitus has been associated with damage to the reproductive system. The present study examines effects of diabetes mellitus on the reproductive system in alloxan-induced diabetic mice after one cycle of spermatogenesis.

Materials and Methods: Twelve (12) mice divided into "control" and "diabetic" groups of six (6) animals each were used. Diabetes was induced in adult male mice by intraperitoneal injection with a single dose of 220 mg/kg body weight of alloxan. After 40 days, sperm density, morphology, and motility were assessed by standard methods. Serum levels of testosterone, FSH, and LH were measured. In addition, testes and epididymis were removed for histological study.

Results: Highly significant ($P < 0.001$) reductions in serum testosterone, FSH, and LH levels, as well as normal and motile sperm counts of 21% and 34% respectively were observed in the diabetic group. The control group had three times more sperm than the diabetic group. The histological

study showed that diabetic animals had atrophied seminiferous tubules, increased inter-tubular spaces, loss of interstitial tissue, degeneration of seminiferous tubules, and almost complete reduction of sperm count in the epididymal duct.

Conclusion: Chronic hyperglycemia is therefore deleterious to the male reproductive system of mice. It leads to hypogonadism, which causes dysfunction of the male reproductive system, and can lead to infertility in men with diabetes mellitus.

Keywords: Reproductive System, Diabetes Mellitus, Spermatogenesis

Introduction

Diabetes mellitus is the most common endocrine disease and one of the most common chronic disorders (Wherrett *et al*., 2018). It is a condition characterized by chronic hyperglycemia (Punthakee *et al*., 2018). The International Diabetes Federation (IDF) estimates that 463 million people were living with diabetes mellitus in 2019. This number is expected to rise to 578 million by 2030 and 700 million by 2045 (IDF, 2019). Poor management of high blood glucose in people with diabetes have led to complications that can significantly reduce their quality of life. Diabetes mellitus has been associated with reproductive system impairment in both men and women (Shojaeii *et al*., 2014). However, a recent study showed that hypogonadism is common in men with diabetes mellitus, and the prevalence is as high as 40% for type 2 diabetes (Bebb *et al*., 2018). This deficit in sex hormone secretion is thought to be responsible for the erectile dysfunction and spermatogenesis disturbance observed in diabetic men (Jangir & Jain, 2014; Ding *et al*., 2015). Hypogonadism has been shown to negatively impact the quality of life of men, irrespective of age, with a greater impact in men with permanent, rather than intermittent, erectile dysfunction (Rosen *et al*., 2004; Corona *et al*., 2013; Maiorino *et al*., 2017). In addition, diabetic men with hypogonadism have an increased risk of cardiovascular mortality compared to eugonadal men with diabetes (Bebb *et al*., 2018).

Although hypogonadism is well recognised as a complication of diabetes, infertility among diabetic men is a lesser examined problem, and the assessment of gonadal status is not clearly established. Alloxan-induced diabetic mice are widely used to study the effect of diabetes mellitus on fertility (Carvalho *et al*., 2003; Arikawe *et al*., 2006; Gumieniczek & Wilk, 2009). The present study examines effects of diabetes mellitus on the male reproductive system of alloxan-induced diabetic mice after one cycle of spermatogenesis (34 days for mice). Specifically, it aims to analyse serum levels of testosterone and pituitary gonadotropins, sperm parameters, testicular weight, and the condition of testicular and epididymal tissue.

Materials and Methods

Experimental Animals

Adult male Swiss mice were used in this study. They were 10 to 12 weeks old and weighed between 28 and 32 g. These animals were bred in the vivarium of the "École Normale Supérieure" in Abidjan (Ivory Coast). They were housed and maintained at a constant temperature of 27-29º C with a relative humidity of 65% and standard 12:12 h light-darkness cycles. Animals were handled according to the guidelines of the Ethical Committee on the use and care of experimental animals of the Department of Biosciences, Université Félix Houphouët-Boigny, Ivory Coast.

Induction of Diabetes

Animals were deprived of food for 16 hours. Diabetes was induced by intraperitoneal injection of a single dose of 220 mg/kg body weight (BW) of alloxan dissolved in isotonic solution (0.9% NaCl). Animals developed diabetes after 3 days. Mice with blood glucose level of 3 g/L (clinical diabetes ≥ 1.26 g/L) or higher were selected for the study.

Experimental Design

Mice were divided into two (2) groups of six (6) animals as follows:

- Group 1: Healthy mice (control);
- Group 2: Diabetic mice.

They were housed in groups of 3 animals. They had free access to standard rodent chow and tap water ad libitum for 40 days. At the end of the experiment, animals were anesthetized with ether in order to collect spermatozoa for analysis of sperm parameters. Blood samples were collected in dry tubes for the determination of serum testosterone, FSH, and LH concentrations. In addition, testes and epididymis were immediately removed, weighed, and fixed in 10% formalin for histological study.

Blood Glucose Level and Body Weight Measurements

Animals were fasted prior to the determination of blood glucose level and body weight. Values were recorded weekly, and blood glucose estimation was performed with an On Call® Extra test strip meter (USA). Blood samples were collected from the tail end of the mice.

Sperm Collection

Animals were anesthetized with ether. The tail of the left epididymis was collected by opening the scrotum, and then dilated in 5 mL of 9‰ NaCl previously incubated in a water bath at 36 °C. Thus the spermatozoa diffused into the solution (Ngoula *et al*., 2007).

Sperm Motility

A fine drop of epididymis macerate was placed and spread lightly on a slide previously maintained at 36°C. The slide was mounted on a light microscope (Olympus CX31RBSF, Philippine) at ×100 magnification. The sperm were filmed with an AmScope (SN: 1605261081) camera (London, United Kingdom). Motile and immobile sperm were subsequently counted on 5 random fields, and the percentage of motile forms was determined (Zougrou *et al*., 2018).

Sperm Cell Concentration

A drop of epididymis macerate was collected and deposited on a Malassez cell and covered with a cover slip. The sperm count was performed under a light microscope (magnification ×400). The number of sperm per mm^3 was estimated by the following formula (Sultan *et al*., 1982):

$$N = \frac{X \times fd \times 10^6}{4}$$

X: Number of sperm counted in 5 grids of the Malassez cell
fd: Dilution factor (20)
N: Number of sperm per mm^3

Sperm Morphology

Sperm morphological abnormalities include fusion, isolated heads, and deformed heads and/or tails (OECD 416, 2001). Two hundred (200) sperm were examined in liquid medium on 3 random fields. The percentage of normal sperm was calculated (Linder *et al*., 1992).

Serum FSH, LH and Testosterone Measurements

Pituitary gonadotropins (FSH and LH) and testosterone were determined using the Hitachi 902 (Japan) ELFA (Enzyme Linked Fluorescent Assay) technique.

Histological Study

Testicles and the right caudal epididymis were removed and fixed in 10% formalin. After 72 hours, they were dehydrated and cleared in alcohol (80°, 90° and 100°) and toluene (99.5 %) baths respectively. Each organ was impregnated and embedded in paraffin. The whole set was cut at 5 µm with a microtome (Leica RM2125 RTS, Germany). The resulting sections were stained in Harris haematoxylin and eosin solutions respectively. Mounting them using Eukitt allowed their good readability under a light microscope (Olympus CK41SF, Philippines) (Zougrou *et al*., 2018). The installation of a

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camera connecting the microscope to a computer allowed image taking via AmScope 3.7 software (London, United Kingdom).

Statistical Analysis

Different values obtained were expressed as the mean, followed by the standard error of the mean (M±SEM). The significance of differences observed between groups of animals was assessed by the Student's T-test using GraphPad Prism 7.03 software (San Diogo California, USA).

Results

Changes in Blood Glucose Concentration and Body Weight

Figure 1 shows the time course of basal blood glucose levels in control and diabetic groups. At the beginning of the experiment, the basal blood glucose levels of the diabetic mice (3.62 ± 0.13 g/L) were significantly elevated ($P < 0.001$) compared to the control (0.70 ± 0.01 g/L). These blood glucose levels remained statistically stable until the end of the experiment. The body weight of control animals increased during the experiment while that of the diabetic mice decreased. Control mice had a weight gain of 21.62% compared to a loss of 27.46% ($P < 0.001$) in diabetic mice (Figure 2).

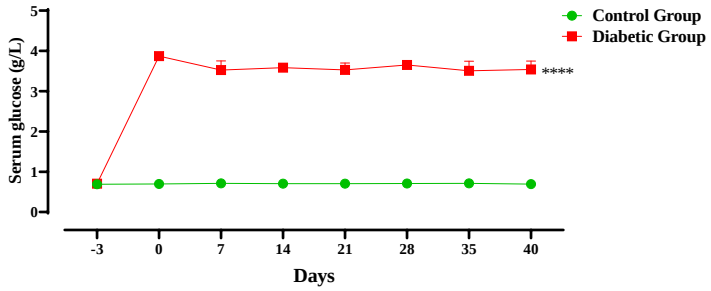

Figure 1. Changes in Serum Glucose Concentration in Experimental Groups during the Experiment
Comparison control group and diabetic group, (Mean±SEM), (n = 6). **** p<0.0001

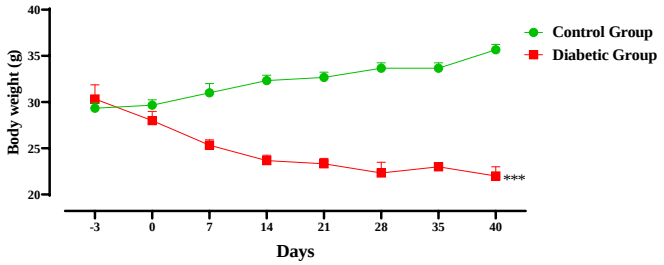

Figure 2. Changes in Body Weight in Experimental Groups during the Experiment
Comparison control group and diabetic group, (Mean±SEM), (n = 6). *** p<0.001.

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Testicular Weight and Serum FSH, LH and Testosterone Levels

Testicular weights of diabetic animals (0.68 ± 0.03 g) were significantly lower (P < 0.5) than those of healthy animals (0.79 ± 0.02 g), a decrease of 16% (Figure 3). Diabetic mice had significantly (P < 0.001) lower levels of sex hormones than the control group. Indeed, the serum testosterone level of the diabetic group was 24.28 times lower than that of the control group. The FSH and LH levels of these diabetic animals were 4.15 and 3.93 times lower than those of the control group respectively (Table I).

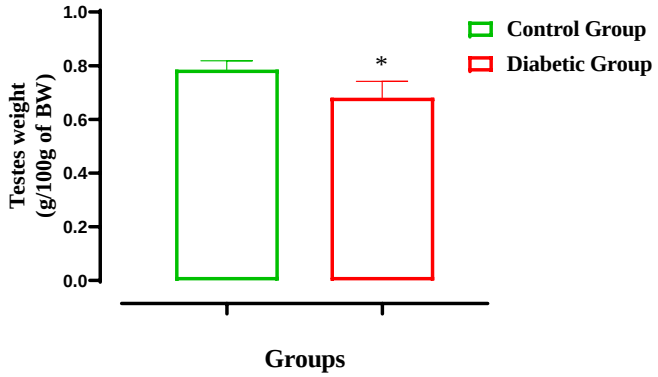

Figure 3. Testes Weight in Experimental Groups
Comparison between control group and diabetic group, (Mean±SEM), (n = 6).
*p< 0.05

Table I. Blood Concentrations of Testosterone, FSH and LH in Experimental Groups

	Groups	
	Control group	**Diabetic group**
Testosterone (ng/mL)	1.603 ± 0.077	0.066 ± 0.008 ****
FSH (UI/mL)	0.137 ± 0.007	0.033 ± 0.003 ***
LH (UI/mL)	0.247 ± 0.009	0.063 ± 0.007 ****

Comparison between control group and diabetic group, (Mean±SEM), (n = 6). *** p<0.001, **** p<0.0001

Sperm Parameters

The control group had 3 times more sperm than the diabetic group (Figure 4). Significant (P < 0.001) reductions of 21% and 34% in normal and motile sperm counts respectively were observed in diabetic mice compared to healthy mice (Figures 5 and 6).

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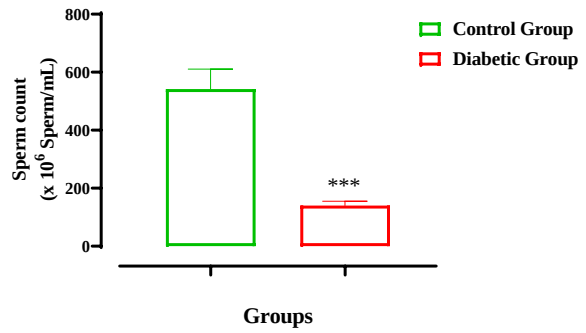

Figure 4. Sperm Count in Experimental Groups
Comparison control group and diabetic group, (Mean±SEM), (n = 6). *** p<0.001

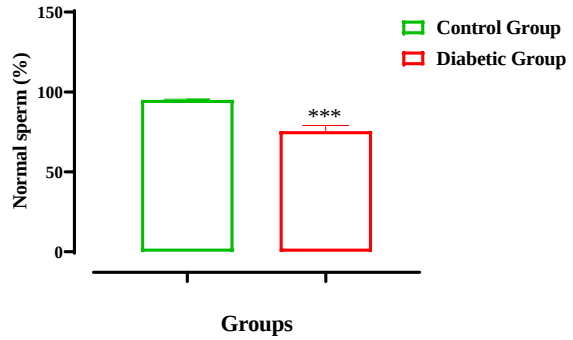

Figure 5. Normal Sperm in Experimental Groups
Comparison control group and diabetic group, (Mean±SEM), (n = 6), *** p<0.001

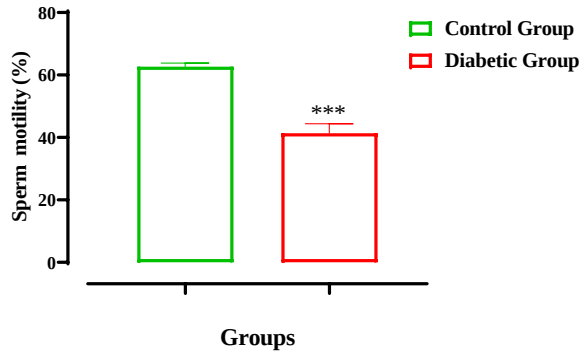

Figure 6. Sperm Motility in Experimental Groups
Comparison control group and diabetic group, (Mean±SEM), (n = 6). *** p<0.001

Histological Study

Figure 7 shows cross sections of testes and epididymis of healthy and diabetic mice. Seminiferous tubules of healthy mice were intact. Different stages of spermatogenesis were observed. Interstitial tissue was present. In

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contrast, seminiferous tubules of diabetic animals were atrophied. There was also an increase in inter-tubular spaces, loss of interstitial tissue, and degeneration of seminiferous tubules. As for the epididymis, an almost complete reduction in the number of spermatozoa in its duct was observed in diabetic animals compared to healthy animals.

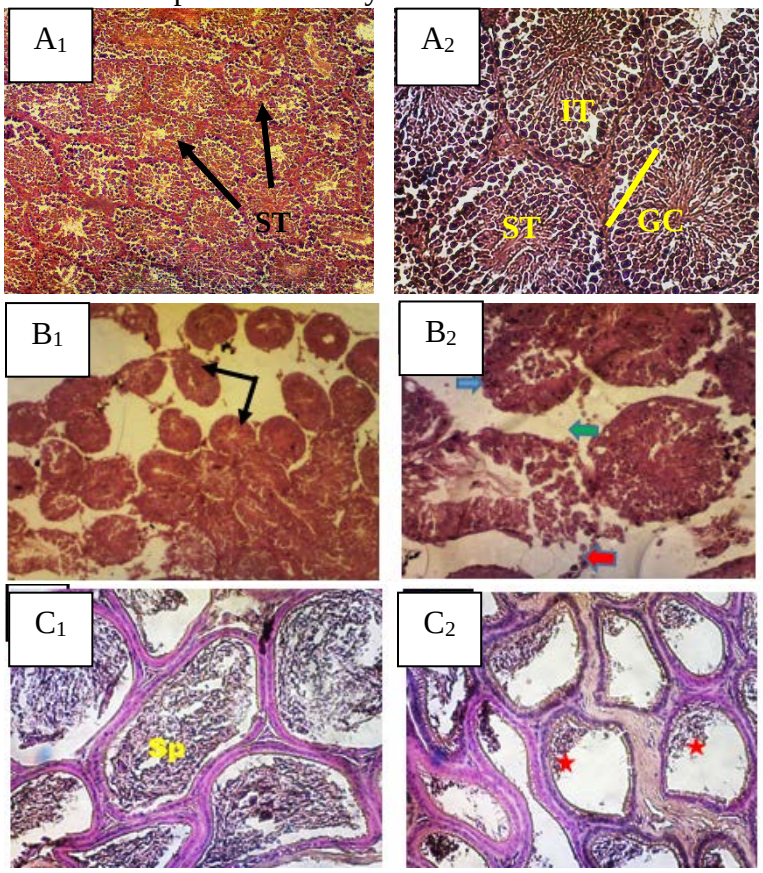

Figure 7. Cross Section of Testicular Tubules and the Epididymis in Experimental Groups A_1 and A_2: Seminiferous tubules of the control group; B_1 and B_2: Seminiferous tubules of the diabetic group; C: Epididymis of the control group; D: Epididymis of the diabetic group; ST: Seminiferous tubules; IT: Interstitial Tissue; GC: Germ Cells; Sp: Sperm. Tubular atrophy (blue arrows); Increase of intercellular space (green arrows); Degeneracy of seminiferous tubules (red arrows); reduction of sperm population (red asterisks). *A_1 and B_1 Magnification:×100 ; A_2, B_2, C and D Magnification: ×400 ; Hematoxylin and Eosin Staining*

Discussion

This study was performed to examine effects of diabetes mellitus on the male reproductive system of mice after one cycle of spermatogenesis. Chronic hyperglycemia was induced by alloxan. Alloxan is a drug widely used to induce experimental diabetes. It selectively destroys β-cells of pancreatic

islets (Szkudelski, 2001; Soltésová & Herichová, 2011). In contrast to streptozotocin, it seems that the change that appears in other organs after alloxan administration is related to chronic hyperglycemia and not to alloxan (Bolzan & Bianchi, 2002). In this study, the blood glucose level of the diabetic group was significantly higher than the control group. This is due to a decrease in serum insulin levels as a consequence of the destruction of pancreatic islet β-cells. In addition, the body weight of diabetic animals decreased during the experiment while that of the control group increased. This weight loss can be explained by an increase in gluconeogenesis in diabetic subjects. Indeed, insulin deficiency leads to an increase in serum levels of counter-regulatory hormones (glucagon, catecholamines, cortisol and growth hormone) (Meyer *et al*., 1998). These hormones induce lipolysis and lead to an increase in the serum concentration of gluconeogenesis precursors.

Serum FSH, LH and testosterone concentrations in the diabetic group were significantly lower than in the control group. This result is consistent with the literature (Murray *et al*., 1981; Steger *et al*., 1989; Ballester *et al*., 2004). Yogev *et al*. (1985) even noted a systematic decrease in serum LH level in diabetic animals from the second day of diabetes. The reduction in pituitary gonadotropin level is thought to be due to a decrease in GnRH level. Indeed, results of a GnRH stimulation test on the pituitary in diabetic rats showed a blunted response (Seethalakshmi *et al*., 1987).

Thus, a decrease in the expression of LH associated with that of insulin in the testicular tissue leads to a dysfunction of the Leydig cells, and thus a defect in testosterone secretion. Hypogonadism would therefore be one of the potential causes of the decrease in sperm count and normal mobile sperm rates observed in the diabetic group. However, oxidative stress caused by chronic hyperglycemia is thought to be the major cause of testicular damage. Indeed, previous work indicated that oxidative stress damages sperm nuclear and mitochondrial DNA (Amaral *et al*., 2006; Aitken & Kopper, 2011; Kotian *et al*., 2019). Spermatozoa are highly vulnerable to oxidative attack as they lack significant antioxidant protection due to the limited volume and distribution of cytoplasmic space in which an appropriate armoury of defensive enzymes is housed (hyaluronidase, acid proteinases, arylsulfatase, ribonuclease, alkaline phosphatase). In particular, sperm membrane lipids are sensitive to oxidative stress because they are rich in polyunsaturated fatty acids. The susceptibility to oxidative attack is further exacerbated by the fact that these cells actively generate reactive oxygen species (ROS) to stimulate the increased tyrosine phosphorylation associated with sperm capacitation.

However, this positive role for ROS is reversed when sperm are stressed. Under these conditions, they default to an intrinsic apoptotic pathway characterised by mitochondrial ROS generation, loss of mitochondrial membrane potential, caspase activation, exposure to phosphatidylserine, and

oxidative DNA damage (Aitken *et al*., 2016). Thus, cessation or dysfunction of spermatogenesis leads to reduced sperm count and increased numbers of abnormal and immobile sperm. Delfino *et al*. also showed that diabetes mellitus alters sperm parameters and affects sperm quality (Delfino *et al*., 2007). The alteration of sperm parameters is corroborated by the analysis of histological sections of the testes and epididymis of diabetic animals. Indeed, seminiferous tubules of diabetic animals were atrophied. There was also an increase in inter-tubular spaces, a loss of interstitial tissue, and degeneration of seminiferous tubules. An almost complete reduction in sperm count was observed in the duct of the epididymis. Thus, the decrease in testicular weight is related to the decrease in the number of germ and somatic cells in testicles. Comparable results have been reported by Shojaeii *et al*. and Kianifard (Shojaeii *et al*., 2014; Kianifard, 2016).

Conclusion

In conclusion, chronic hyperglycemia is deleterious to the male reproductive system of mice. Hypogonadism, which might be associated with oxidative stress and caused by this persistent hyperglycemia, induce a decrease in sperm count, normal and motile sperm counts and testes weight, alteration of sperm parameters, atrophy, and degeneration of seminiferous tubules. This can lead to infertility in men with diabetes mellitus (by extension of this study to men).

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