Original Article

Experimental diabetes and the epididymis of Wistar rats: The protective effects of *Anacardium occidentale* (Linn.)

Victor O. Ukwenya, Olumide J. Ashaolu[1], David O. Adeyemi[2], Korede J. Abraham[3]

Department of Anatomy, College of Medicine, Ekiti State University, Ado-Ekiti, [1]Department of Anatomy, College of Medicine, Bowen University, Iwo, [2]Department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife, [3]Department of Anatomy, Faculty of Medicine, Kogi State University, Anyigba, Nigeria

Abstract

Aim: The use of botanical remedies as adjunct therapies in the management of diabetes mellitus is on the increase. *Anacardium occidentale* has been reported in the literature to possess anti-diabetic and hypoglycemic properties. This study evaluated the effects of acute treatment of *A. occidentale* on blood glucose and epididymis histopathology of streptozotocin (STZ)-diabetic rats. **Materials and Methods:** Forty adult male Wistar rats were randomly divided into 4 experimental groups of 10 rats each. A group served as the normoglycemic control and was administered 1 ml/kg bw/day citrate buffer. Hyperglycemia was induced in 30 overnight-fasted rats with a single i.p injection of STZ (70 mg/kg bw/day). Hyperglycemia was confirmed 48 h later and thereafter allowed to stabilize for 5 days. 300 mg/kg bw/day of ethanolic extract of *A. occidentale* was administered orally to a group of diabetic rats (n = 10). Insulin was also administered subcutaneously at 10 I.U/kg bw/day to another group (n = 10). Another group served as the hyperglycemic control and received 1 ml of citrate buffer/kg bw/day. Treatment after a 5-day stabilization of hyperglycemia lasted for 17 days. In each group blood glucose and epididymal histology were assessed. **Results:** By the end of the experimental period, all hyperglycemic rats in the extract-treated group had become normoglycemic. Moreover, extract-treated rats showed improved epididymal morphology and luminal sperm aggregate within the duct comparable to normoglycemic and insulin-treated rats. **Conclusion:** We conclude that *A. occidentale* proved valuable in mitigating the detrimental effects of hyperglycemia on the epididymis.

Key words: Diabetes mellitus, hyperglycemia, reticulin fibers, sperms, streptozotocin

Address for correspondence:
Dr. Victor O. Ukwenya,
Department of Anatomy, College of Medicine, Ekiti State University, Ado-Ekiti, Nigeria.
E-mail: victorwyn@yahoo.com

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INTRODUCTION

Diabetes mellitus (DM) is a group of syndromes characterized by hyperglycemia, altered metabolism of lipids, carbohydrates and proteins, and an increased risk of complications from vascular diseases which are attributed to an insufficient supply of insulin (Davis, 2006).

Diabetes mellitus is an epidemic metabolic disease concurrent with falling fertility rates and severe detrimental blood testis barrier (BTB) alterations. It induces testicular alterations, disrupting the metabolic cooperation between the cellular constituents of BTB,

with dramatic consequences on sperm quality and fertility (Alves *et al*., 2013).

The epididymis is an important organ of male reproduction which imparts sperm maturation, acquisition of motility, and fertilizing potential as well as removal of defective sperm and providing a good microenvironment (Turner *et al*., 2007). In addition to sperm maturation, the epididymis plays a significant role in the transport, concentration, protection and storage of spermatozoa, and all these process are androgen dependent (Meistrich, 1975).

Diabetes mellitus may affect male reproductive function at multiple levels including spermatogenesis as a result of its endocrine impairment (Baccetti *et al*., 2002; Ballester *et al*., 2005).

Animal studies using rodent models of streptozotocin (STZ)-induced DM have demonstrated a reduction in sperm count and quality (Amaral *et al*., 2006; Scarano *et al*., 2006). Human diabetic study showed reduction in all semen parameters (semen volume, sperm count, motility, and morphology) (García-Díez *et al*., 1991). This reduction in spermatogenesis and seminal parameters were secondary to diminished testosterone production.

Several studies have highlighted the detrimental effects of diabetes on the epididymis. Diabetes-related alterations in epididymal cells are also related to changes in the pituitary–testicular axis (Steger and Rabbe, 1997). A study by Suresh *et al*. (2011) revealed major damage in structure of epididymis and altered sperm quality and quantity under the influence of long-term diabetes. Macroscopic changes reported after induction of experimental diabetes included reduction in epididymal weight (Priyadarshani and Varma, 2014).

Present treatment regimen for DM includes the use of oral hypoglycemics, exogenous insulin, transplantation, and xenotransplantation. Current drug therapy, however, does not provide sufficiently tight control of blood glucose to avoid diabetes complications (Serup *et al*., 2001) and no satisfactory effective therapy is yet available to cure DM (Mallick *et al*., 2006).

The setbacks being encountered in present anti-diabetic therapies call for innovative treatment therapies that are effective, less toxic, and less expensive compared to synthetic drugs.

The emerging field of nutraceuticals has further highlighted the importance of plant use in medicine. Nutraceuticals comprise phytochemicals from plants such as terpenoids, lignins, saponins, circumins, isoflavones, plant steroids, flavonoids, and indoles (Whitman, 2001).

Anacardium occidentale (Linn.) has been described to possess hypoglycemic activities among others. The hypoglycemic property of the inner stem of *A. occidentale* in alloxan-diabetic rats have been described (Abdullahi and Olatunji, 2010). The anti-diabetic and anti-inflammatory properties of the leaf and bark extracts of the cashew plant have also been validated (Mota, 1985; Kamtchoury, 1998; Esimone *et al.*, 2001). The stem-bark and leaves of *A. occidentale* have been reported to possess hypoglycemic activities (Ojewole, 2003).

Previous studies on the anti-diabetic effects of *A. occidentale* focused mainly on the testes and seminal parameters. The objective of the present study was to analyze the morphological changes in the epididymis under hyperglycemic condition and to determine if the methanolic extract of *A. occidentale* has any differential effects on the epididymal tissue of diabetic rats model.

MATERIALS AND METHODS

Animal Care

Forty presumably healthy and normoglycemic adult male Wistar rats of average weight 150–175 g were used for this study. The animals were kept in iron cages at controlled room temperature of about 30°C and photo-periodicity of 12L: 12D in the Animal House of the Department of Anatomy, Bowen University, Iwo, Nigeria. They were allowed free access to rat chow and water.

Extract Preparation

Fresh leaves of *A. occidentale* were collected from Iwo, Osun state of Nigeria, and identified by a plant taxonomist in the Department of Plant Biology, University of Ilorin, Ilorin. The voucher specimen (UIH/612) was deposited in the departmental herbarium.

The leaves of *A. occidentale* were washed and shade-dried to constant weight and then ground into fine powder using a contact mill (Tower, Italy). A volume of 1977.7 g of the leaf powder was percolated in 4720 ml of methanol for 48 h and then filtered through Whatman filter paper at room temperature. The supernatant was concentrated under reduced pressure using a rotary evaporator (Labarato 4000, Bibby Sterilin, China). The final product was a dark green sticky mass weighing 89.8 g, which represented a yield of 4.54%. The concentrate was freeze-dried, kept in a dessicator and refridgerated at 4°C before use.

Stock solution was prepared by dissolving 42 g of the extract in 265 ml of physiological saline to give a concentration of 0.16 g/ml. The stock solution was refridgerated.

Experimental Protocol

Diabetes was induced in the rats by a single intraperitoneal injection of 70 mg/kg body weight of STZ in 0.1 M citrate buffer at pH 4.5. Accu-Check Glucometer (Roche) and compatible glucometer strips were used for the determination of blood glucose levels in over-night fasted rats 48 h after induction of hyperglycemia. Blood samples were obtained from the dorsal vein of the tail of conscious rats. Only rats with glucose levels >250 mg/dl were recruited into the study. Hyperglycemia was allowed to stabilize for 5 days after the confirmation of hyperglycemia before the commencement of extract and insulin administration. The rats were randomly divided into three experimental groups of 10 animals each: Hyperglycemic control (HC), administered 1 ml/kg bw/day citrate buffer; hyperglycemic + 300 mg/kg bw/day of extract (HE), hyperglycemic + 1.0 I.U/kg bw/day insulin (HI). Another group received only 1 ml/kg bw/day of citrate buffer (placebo) and served as the normoglycemic control (NC).

For groups HC, HE, and HI, treatment lasted for 17 days. Thereafter, the blood glucose level of all animals in each experimental group was assessed every other day after treatment with the extract and protamine zinc insulin (Insulatard®, Novo Nordisk, Denmark).

The procedures involving animal use in this study were in compliance with the guiding principles for research involving animals as stipulated by the declaration of Helsinki (American Physiology Society, 2002) and were approved by the departmental committee on the use and care of animals.

Tissue Harvesting and Processing

Twenty four hours after the administration of the last doses of drug and extract, the animals were weighed and glycaemia was determined by means of a glucometer (Accu-Check, Roche). After sacrifice by cervical dislocation, the animals were opened up by abdominopelvic incision and the epididymides were excised, blotted dry, and fixed in Bouin's fluid. The fixed tissues were transferred to graded series of ethanol and then cleared in xylene. The tissues were then infiltrated in two changes of molten paraffin wax at 58°C for 1-h in each change and finally embedded in paraffin wax using multi-block plastic embedding molds. The paraffin blocked tissues were trimmed and mounted on wooden block for sectioning. Serial sections of 5 μm thick were obtained from a solid block of tissue on rotary microtome (Leica Rm 2135, England).The sections were fixed on clean slides, stained with Hematoxylin and Eosin stains as described by Culling (1974) and silver impregnation according to the method of Gordon and Sweet (1936).

Statistical Analysis

Values were compared using computerized software: SPSS 16.0 (Microsoft, 2010 Version, USA). Values were recorded as mean ± standard error of mean (SEM). The statistical significance of difference in the mean and SEM ($P < 0.05$) were analyzed by one-way analysis of variance and the Scheffe's *post-hoc*.

RESULTS

Forty-eight hours after injection with 0.1 M STZ, all treated rats had blood glucose concentrations >250 mg/dl [Table 1]. The NC rats had normal glycemic concentrations below 100 mg/dl; this value was maintained throughout the experimental period [Table 1].

At the end of the experimental period, group HE showed significant reduction in the blood glucose levels ($P < 0.05$) compared to HC rats (102.78 ± 10.75 mg/dl vs. 417.89 ± 33.39 mg/dl). Final blood glucose level for group HI also differed significantly ($P > 0.05$) from HC (112.89 ± 18.32 mg/dl vs. 417.89 ± 33.39 mg/dl [Table 1].

H and E Sections

Sections of the epididymides from groups NC, HE, and HI featured large tubules lined with ciliated pseudostratified epithelium featuring tall columnar principal cells. The tubules were distended with luminal sperm aggregate and were surrounded by a distinct layer of smooth muscle [Figure 1a, c, and d]. Sections from the HC rats showed tubules with ciliated pseudostratified columnar epithelium with distinct principal and basal cells [Figure 1b]. The tubular lamina appeared reduced and contained scanty sperms compared to NC, HE, and HI groups [Figure 1b]. The stroma appeared thicker and the epithelia contracted compared to NC, HE, and HI groups. Adjacent to the tubules is a distinct layer of smooth muscle and connective tissue fibers.

Table 1: Effects of methanolic extract of *Anacardium occidentale* and insulin on the body weight and blood glucose

Groups	Blood glucose concentration (mg/dl)	
	Initial	Final
NC	91.80±3.81	95.90±4.16†
HC	358.64±18.90*	417.90±33.39
HE	333.62±15.19*	102.78±10.75†
HI	339.91±9.99*	112.89±18.32†

Values are mean±SEM; *n*=10 in each group. *Significantly different compared to Group NC at *P*<0.05, †Significantly different compared to HC at *P*<0.05. NC: Normal control rats; administered only 1 ml/kg bw/day citrate buffer (placebo), HC - Hyperglycemic control; administered 70 mg/kg bw/day STZ only, HE - Hyperglycemic+extract-treated rats; administered 70 mg/kg bw/day STZ+300 mg/day of methanolic leaf extract of *Anacardium occidentale*, HI - Hyperglycemic+insulin-treated; administered 70 mg/kg bw/day STZ+1.0 I.U/kg bw/day insulin, SEM - Standard error of mean, STZ - Streptozotocin

Silver Impregnation

Silver impregnation revealed the presence of extensive reticulin fibers around the peritubular walls of NC, HE, and HI groups. The connective tissue stroma and tunica vaginalis of these groups also revealed a dense network of reticulin fibers [Figure 2a, c, and d].

Silver impregnation showed sparse reticulin fibers within the stroma and peritubular walls of the epididymis of HC rats [Figure 2b]. Visible within the tunica vaginalis are fragmented strands of reticulin fibers [Figure 2b].

DISCUSSION

The epididymis is the site of sperm storage and maturation. In this study, the epididymis of NC, HE, and HI appeared normal, with the tubule distended with sperms. Those of the HC had smaller lumen, had undergone shrinkage, which made the epithelial cells, appeared markedly columnar with the principal cells clumped together. More importantly, the tubular lumen was devoid of spermatozoa. These reflect damage caused by hyperglycemia and supports similar findings in STZ-diabetic prepubertal rats. Soudamani *et al*. (2005) reported a considerable reduction in the size of the tubule and lumen of the epididymis, tightly packed principal cells with clumped nuclei and lumen devoid of spermatozoa. The evidence from this present study implies that the effects of STZ on the epididymis are not age-dependent.

The increased thickness in the connective tissue stroma of untreated hyperglycemic (HC) rats observed in this study [Figure 1b] also agrees with the findings of Fernandes *et al*., (2011), who reported significant increase in epididymal stromal compartment and duct epithelium.

Previously, we demonstrated the presence of reticulin fibers within the epididymis of adult male Wistar rats (Ukwenya *et al*., 2014). This study demonstrated the impact of experimental diabetes on the reticulin fibers within the epididymis. The epididymides of NC, HE, and HI rats featured abundant reticulin fibers. The reticulin fibers formed a wavy meshwork that extended from the tunica vaginalis and ramified within the connective tissue. They were also present within the peritubular walls of the epididymides. On the other hand, the epididymides of HC rats displayed reticulin fibers mainly around the peritubular walls. The tunica vaginalis and connective tissue showed poor localization and intensity for the fibers.

Reduction of these fibers within the connective tissue would exert a negative effect on contractility, affecting transit time of sperm within the epididymis, since transport of mature spermatozoa through the remainder of the male reproductive system is achieved via muscle contraction rather than the spermatozoon's recently acquired motility (Ning *et al*., 2011). In fact, diminished reticulin fibers noted in this study could be responsible for the lower transit time of epididymal sperms reported by Fernandes *et al*., (2011). Diminished secretion of reticulin fibers would also have a deleterious impact on maturation, as it has been long proven that the key event in sperm maturation is not the passage of time but the exposure of the sperm to the luminal environment of the epididymis (Bedfort, 1967).

In general, the epididymis depends on androgens to maintain their structure and function (Amman *et al*., 1993). The morphological alteration seen in diabetic epididymis may be predominantly due to the androgen deficiency. Hypogonadism has been associated with diabetes in both human (Baccetti *et al*., 2002) and rat subjects (Altay *et al*., 2003). Significant reduction in plasma and serum testosterone concentration have been reported in diabetic rats (Arikawe *et al*., 2006; Khaneshi *et al*., 2013).

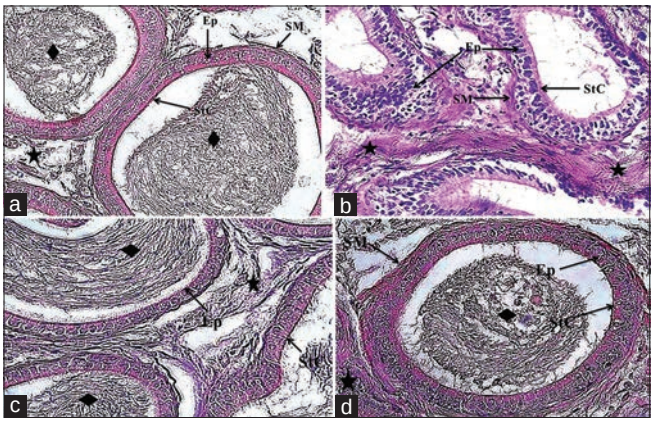

Figure 1: Cross-section of epididymal tubules of adult Wistar rats of (a) normal control, (b) hyperglycemic control, (c) hyperglycemic + extract-treated, (d) hyperglycemic + insulin-treated (H and E, ×400). Rhomboids indicate luminal sperms; stars indicate connective tissue (Ep: Epithelium; StC: Stereocilia; SM: Smooth muscle)

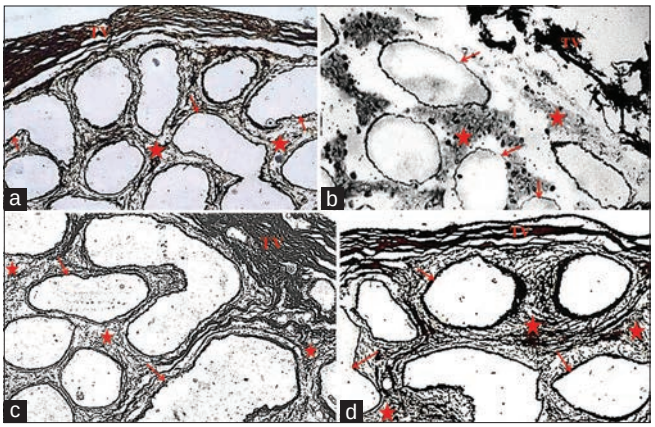

Figure 2: Cross-section of epididymal tubules of adult Wistar rats of (a) normal control, (b) hyperglycemic control, (c) hyperglycemic + extract-treated, (d) hyperglycemic + insulin-treated (Gordon and Sweet's, ×100). Arrows indicate peritubular walls; stars indicate connective tissue (TV: Tunica vaginalis)

One of the detrimental effects of hyperglycemia on reproductive function is oligospermia. Results from this study showed that the reduced sperm count encountered in diabetics is due mainly to hypospermatogenesis induced by testicular lesions and damage to the epididymis. Results also showed that the methanolic leaf extract of *A. occidentale* possesses anti-diabetic properties comparable to insulin in reversing the histopathological features induced by hyperglycemia. This might be due to the anti-oxidant properties of the plant material. Phytochemical study of the methanolic leave extract revealed the presence of the potent anti-oxidants, phenolic, and flavonoids (Fazali *et al*., 2011).

We conclude that hyperglycemia has a negative effect on epididymal morphology and sperm quantity; and that *A. occidentale* proved valuable in mitigating the detrimental effects of hyperglycemia on the epididymis.

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