Ocimum gratissimum leaf extract may precipitate infertility in male diabetic Wistar rats

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ABSTRACT
Objective: This study was designed to investigate the Ocimum gratissimum (OG) effects on sperm quality and testicular cytoarchitecture in alloxan-induced diabetic rats.

Method: Twenty male Wistar rats (150-200 g) were assigned into 4 groups (n=5) as A (control), B (OG), C (Dia) and D (Dia+OG). Groups A and B were normal animals receiving distilled water or OG (400 mg/kg), respectively while diabetes was induced by alloxan monohydrate (100 mg/kg) in groups C and D, followed by the administration of distilled water or OG, respectively for 28 days. Blood samples were obtained for fasting blood glucose (FBG) and fructosamine determination while, epididymis and testes were obtained for sperm quality assessment using computer-assisted sperm analyzer and testicular histomorphometry, respectively. Seminiferous tubule diameter and interstitial space distance were quantified in hematoxylin and eosin stained slides. Statistical analysis was done using ANOVA and student t-test at p<0.05.

Results: Fructosamine and FBG were reduced in Dia+OG (80.11±3.80µmol/L and 132.0±8.41mg/dl, respectively) compared with Dia (139.66±4.29µmol/L and 285.6±26.69mg/dl, respectively). Sperm count was unchanged in Dia, but decreased in OG and Dia+OG; abnormal sperm cells increased in OG, Dia and Dia+OG. Mild vacuolation in the seminiferous tubule, disorganized germinal cells layer, arrested sperm maturation with empty spermatooza in lumen, decreased seminiferous tubule diameter and increased interstitial space were found in the testes of OG, Dia and Dia+OG compared with control.

Conclusion: Diabetes induces sperm impairments and distortions in testicular cytoarchitecture, which were aggravated by OG leaf extract in male Wistar rats.

Keywords: Ocimum gratissimum, fructosamine, sperm quality, testicular cytoarchitecture

INTRODUCTION

Sexual dysfunctions ranging from erectile and testicular dysfunctions, reduced libido, retrograde ejaculation (Dunsmuir & Holmes, 1996), disrupted endocrine control of spermatogenesis (Baccetti et al., 2002), impaired sperm DNA integrity (Agbaje et al., 2007), reduced sperm count and motility (Barták, 1979; Bhattacharya et al., 2014) and low serum testosterone (Verma et al., 2013) are complications of prolonged diabetes mellitus in men. Thus, a high prevalence of infertility and subfertility is associated with type 1 and type 2 diabetes mellitus (Alves & Oliveira, 2013; Rato et al., 2013). The prevalence could be as high as 35% in type 2 diabetes mellitus (La Vignera et al., 2012a), with about 90% of diabetics experiencing sexual upheavals such as decreased libido, impotence and infertility (Corona et al., 2014). With the growing incidence of diabetes mellitus, which had already surpassed the World Health Organization’s projection for 2025 by 28.9% in 2014 (Temidayo & Stefan, 2018), infertility among diabetic men is likely to grow in similar progression.

Data from experimental diabetes mellitus have also shown that uncontrolled hyperglycaemia may be deleterious to the male reproductive function. For instance, decreased copulatory behavior (Scarano et al., 2006), reduced fertility, decrease Leydig cells population (Ballester et al., 2004) and increased testicular toxicity (Hadi et al., 2013; Iweala et al., 2013) were reported in diabetic rats. Also, in prediabetes induced by high energy diet in rats, alterations in testicular glucose metabolism and epididymal bicarbonate dynamics may adversely affect sperm storage and viability (Rato et al., 2013). The testicular dysfunction/ degeneration in experimental diabetes was hypothesized to involve oxidative stress (Amaral et al., 2006; Shrilatha & Muralidhara, 2007; La Vignera et al., 2012b). Increased oxidative stress is associated with increased sperm DNA damage and spermatogenic gene expression (Malilidis et al., 2007, 2009); hence, treatment with different antioxidants were shown to improve diabetes-induced sperm abnormality in animal models (Rabbani et al., 2009; Bal et al., 2011; Mohasseb et al., 2011; Shi et al., 2017; Tsounapi et al., 2018) and men (Omu et al., 2014). The lack of insulin stimulatory effect on Follicle Stimulating Hormone (FSH) in experimental type-1 diabetes may also play a role in the decreased Leydig cell function and testosterone production (Ballester et al., 2004); given that insulin replacement significantly improved sperm quality and testicular cytoarchitecture in diabetic rats (Seethalakshmi et al., 1987; Soudamani et al., 2005). Ocimum gratissimum (OG), planted in Nigeria for its nutritional and medicinal value, has been shown by several researchers to possess hypoglycaemic (Aguiyi et al., 2000; Owoyele et al., 2005; Egesie et al., 2006) and antioxidant (Akinmoladun et al., 2007; Aprioku & Obianime, 2008; Shittu et al., 2016) properties. The hypoglycemic property was associated with inhibition of hepatic glycogen phosphorylase activity in streptozotocin-induced diabetic rats (Shittu et al., 2018). Current evidences in normal rats (Leigh & Fayemi, 2008) and mice (Obianime et al., 2010) have documented that OG may possess anti-fertility properties in a dose and duration dependent manner. However, there are conflicting reports on the influence of OG on male reproductive parameters in diabetic rats. For instance, Arfa & Rashed (2008) reported an elevated testosterone level, while Ebong et al. (2014) observed no changes in reproductive hormones in OG-treated diabetic rats. Also, Asuquo et al. (2009) reported improvements in testicular morphology, while Onuka et al. (2014) reported impaired sperm parameters in OG-treated diabetic rats. It is therefore pertinent to investigate the effects of OG on sperm quality and testicular cytoarchitecture in alloxan induced diabetic rats.
MATERIALS AND METHODS

Animals

Twenty male Wistar rats were obtained from the Central animal house, College of Medicine - University of Ibadan, Ibadan. They were housed and acclimatized for two weeks in the Department of Physiology animal house, University of Ibadan, under standard laboratory conditions with natural photoperiod of 12 hours light: dark cycle. They were allowed free access to rat chow (Ladokun Feeds) and water. All experimental and handling protocols were in compliance with institutional ethical regulation and the NIH publication No. 85-23 guidelines (NIH publication revised, 1985).

Preparation of aqueous leaf extract of Ocimum gratissimum

Fresh leaves of OG were obtained from the Bode market in the Ibadan metropolis. Identified and authenticated at the Forest Research Institute of Nigeria (FHI.110026). The fresh leaves were washed, air-dried and pulverized into a powder form. One kilogram of the powder was soaked in a glass container with distilled water for aqueous extraction for 24 hours, filtered and the filtrate was collected in a round bottom flask. The filtrate was evaporated using a rotary evaporator to yield 8.51% extract. The extract was administered at 400 mg/kg body weight which had been previously reported to be non-lethal (Aguiyi et al., 2000).

Induction of diabetes mellitus

Diabetes mellitus was induced by intraperitoneal administration of 100 mg/kg body weight of alloxan monohydrate (Sigma®, St Louis, USA). Diabetes was confirmed after 72 hours using a One Touch Ultra glucometer®. Animals with fasting blood glucose ≥200mg/dl were considered diabetic.

Experimental design

The rats were randomly divided into 4 groups (n=5) and treated per os for 28 days as follows:

A. Control: Normal animals administered distilled water daily
B. Ocimum gratissimum (OG): Normal animals administered 400 mg/kg of OG
C. Diabetic Untreated (Dia): Alloxan-induced diabetic rats administered distilled water
D. Diabetic Treated (Dia+OG): Alloxan-induced diabetic rats administered 400 mg/kg of OG

Fasting blood glucose (FBG) was monitored at the start and at the end of the 28 day- treatment. Blood sample for FBG was collected via cardiac puncture for measuring fructosamine levels while the epididymis and testes were collected for sperm quality assessment and determination of testicular cytoarchitecture.

Determination of serum fructosamine

Fructosamine levels were measured using the commercially available fructosamine kit (Fortress Diagnostics Limited®, United Kingdom). The colorimetric test principle is based on the ability of ketoamines to reduce nitroblue tetrazolium-blue to formazan in an alkaline solution. The rate of formazan formation is directly proportional to the fructosamine concentration. Uric acid interference is eliminated by Uricase and a detergent eliminates matrix effects. The rate of reaction is photometrically measured at 546nm. Briefly, 50µL of sample or calibrator was added to 1000µL working reagent made up of potassium phosphate buffer, Nitroblue tetrazolium-blue, Sodium cholate, Potassium carbonate buffer (pH 10.3), Uricase (Arthrobacter species) and detergent. It was mixed and incubated for 9 minutes at 37ºC. The absorbance at 546 nm was recorded immediately (A1) and after exactly 60 seconds (A2). The fructosamine concentration (µmol/L) was calculated as:

\[
\text{Concentration of Calibrator} = \frac{A2 - A1 \text{ Sample}}{A2 - A1 \text{ Calibrator}} \times \text{Concentration of Calibrator}
\]

Sperm Count

The right epididymis was placed in a pre-warmed (37ºC) Petri dish containing 2mL of phosphate buffered saline solution (pH 7.4). The caudal portion was punctured twice with the tip of a scalpel to release sperm, commencing a 3-minute “swim-out” period. After the swim-out, the dish was gently swirled, and a 9µL sample from a relatively dense portion of the sperm cloud was placed onto a counting chamber. The sperm count (millions/ml) was determined using a computer-aided sperm analyser (CASA, JH-6004 Sperm Quality Analyser).

Sperm Morphology

The left caudal epididymis was weighed, minced in a Petri dish containing 2 mL of deionized water and swirled gently. A drop of the dish content was placed on a standard glass microscope slide. The edge of a clean slide was gently dragged across the drop to make a thin layer of sperm cells which was put to air dry. The air-dried smear was fixed in 95% and 50% (v/v) ethanol for 15 minutes and 30 seconds respectively, then rinsed in distilled water for 30 seconds and stained with Harris’s hematoxylin and G-6 orange for 4 minutes and 1 minute, respectively. It was then dipped in ethanol 95% for 2 minutes, before staining with EA-50 green for 1 minute (Papanicolaou). The stained slide was then immersed in a xylene and ethanol mixture in a ratio of 1 to 2, and in 100% xylene for 1 minute each, respectively. It was then drained for 1-2 seconds (Marshall, 1983). Two slides were made from each caudal epididymal tissue. The stained slides were examined under the microscope with a x100-objective lens and x10 eyepieces. Abnormal sperm cells were counted and expressed as a percentage.

Testicular histomorphometry

The testes were immediately fixed in Bouin’s fluid for 24 hours. It was subsequently dehydrated twice in ascending grade of absolute alcohol for one hour, and placed in xylene for 1 hour for clearing. After removing it from xylene, it was placed in a wax bath for one hour and embedded in paraffin wax. It was then trimmed and sectioned at 3-5 micron slices with a microtome. The section was floated with 20% alcohol on water at a temperature of 5ºC (below paraffin wax melting point), picked with a clean grease-free microscope slide and drained for 1 hour. It was then stained with hematoxylin and eosin and mounted. Photomicrographs of the sections were made to observe general morphology and morphological changes. Seminiferous diameter and interstitial space distance were quantified by imageJ software (version 1.49, National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/).

Statistical analysis

The data from each group was expressed as mean ± standard error of the mean (mean±SEM). The data was analyzed using ANOVA and Student t-test. p<0.05 was considered significant. All analyzes were performed using GraphPad prism, version 7.
RESULTS

Effect of Ocimum gratissimum on fasting blood glucose and serum fructosamine

As shown in Figure 1, there was no significant difference in the blood glucose levels of the control and the OG-treated normal animals [Control (90.88±4.29 mg/dl vs. 87.75±2.20mg/dl); OG (90.83±5.49 mg/dl vs. 89.67±4.21 mg/dl)] before and after the 28-day treatment. However, fasting blood glucose level was significantly reduced (α0.05) from 285.6±26.69mg/dl before treatment to 132.0±8.41mg/dl after treatment (p<0.05) in the Dia+OG. The effect of OG on fructosamine is shown in Figure 2. Fructosamine level was significantly increased (α0.05) in the Dia group (139.66±4.29µmol/L) when compared with the controls (38.71±0.85µmol/L); while it was decreased (α0.05) in the Dia+OG (80.11±3.80µmol/L) compared with Dia (139.66±4.29µmol/L).

**Figure 1.** Effects of Ocimum Gratissimum on fasting blood glucose level in normal and diabetic male rats. n=5, *p<0.05 Before vs. After

**Figure 2.** Effects of Ocimum Gratissimum on Serum fructosamine in normal and diabetic male rats. n=5, *p<0.05 Control vs. Dia; #p<0.05 Dia vs. Dia+OG

Effect of Ocimum gratissimum sperm quality

Sperm count decreased significantly (α0.05) in OG, Dia and Dia+OG when compared with the controls (Figure 3). The sperm counts in the Dia group was significantly higher (α0.05) than the counts in the Dia+OG group. As shown in Figure 4, the percentage of abnormal sperm morphology was significantly increased (α0.05) in normal rats treated with OG (42.67±1.67%), diabetic untreated rats (38±1.63%) and diabetic rats treated with OG (46.50±5.95%), when compared with the controls (25.83±1.11%).

**Figure 3.** Effects of Ocimum gratissimum on sperm counts in normal and diabetic male rats. n=5, *p<0.05 compared with Control animals

**Figure 4.** Effect of Ocimum gratissimum on sperm morphology in normal and diabetic male rats. n=5, *p<0.05 compared with Control animals

Effects of Ocimum gratissimum on testicular histomorphometry

The effects of OG on testicular cytoarchitecture of normal and diabetic rats are shown in Figure 5 and Table 1. Mild vacuolation in the seminiferous tubule, disorganized germinal cells layer, arrested sperm maturation with empty spermatozoa in lumen where observed in OG, Dia and Dia+OG animals when compared with their control counterparts. The seminiferous tubule diameter was significantly decreased (α0.05) in OG, Dia and Dia+OG groups when compared with the control animals; while interstitial space distance was significantly increased (α0.05) in OG, Dia, and Dia+OG when compared with the control animals.

DISCUSSION

The objective of the present study was to evaluate the effects of OG on sperm quality and testicular...
Figure 5. Photomicrographs of the testes showing seminiferous tubules (black arrow) and their respective interstitial space (black star box) in (A), Control group shows normal seminiferous tubule with complete sperm maturation, normal germinal cells layer; presence of spermatozoa strand in the lumen and interstitial cells appear normal. (B) OG (C) Dia and (D) Dia+OG groups show mild vacuolation in the seminiferous tubule, disorganized germinal cells layer, arrest sperm maturation with empty spermatozoa in lumen. X 100 cytoarchitecture in male diabetic rats. The reduction in the fasting blood glucose level observed in the diabetic animals treated with OG is consistent with the reported hypoglycemic effect of OG (Aguiyi et al., 2000; Owoyele et al., 2005; Egesie et al., 2006; Mohammed et al., 2007; Oguanobi et al., 2012; Okoduwa et al., 2017). Fructosamine was quantified in the present study to monitor glycemic control in the diabetic rats. Fructosamine is formed by the glycation of primary amine and its subsequent isomerization via the Amadori rearrangement (Armbruster, 1987). It reflects glycemic control over the previous 2-4 weeks (Nagasaka et al., 1988), without any influence of erythrocyte diseases (Koga et al., 2011). Although fructosamine is yet to gain wider use, like glycated hemoglobin (HbA1c) in monitoring diabetes control (Nansseu et al., 2015), studies are pointing to its tendency of outperforming HbA1c (Rendell et al., 1986; Misciagna et al., 2004). The elevated fructosamine level in the diabetic rats of the present study is
in line with the documented effect of experimental diabetes on fructosamine (Petlevski et al., 2001; You et al., 2015; Ren et al., 2017). To the best of our knowledge, this present study is the first to report the effects of OG on fructosamine levels in diabetic rats. This is consistent with the report of Leigh & Fayemi (2008) that aqueous extract of OG has deleterious effects on both spermatogenesis and maturation of spermatozoa at different stages of germ cell development. Nevertheless, impairment in these sperm parameters were also observed in the diabetic untreated group, it is more pronounced in the OG-treated diabetic animals. It is a well-documented phenomenon in which increased sperm abnormality is an indicator of testicular pathology (Câmara et al., 2014).

The decreased sperm count and increased percentage of abnormal sperm cells in all the OG-treated animals of this study indicate that OG poses anti-fertility effects on normal and diabetic rats. This is consistent with the report of Leigh & Fayemi (2008) that aqueous extract of OG has deleterious effects on both spermatogenesis and maturation of spermatozoa at different stages of germ cell development. Nevertheless, impairment in these sperm parameters were also observed in the diabetic untreated group, it is more pronounced in the OG-treated diabetic animals. It is a well-documented phenomenon in which increased sperm abnormality is an indicator of testicular pathology (Câmara et al., 2014).

The decreased mean diameter of seminiferous tubules in the experimental animals suggests that testicular damage caused by OG could be linked to the observed significant changes in sperm parameters, since the histological integrity of the entire testis is fundamental to the production of fertile spermatozoa (White, 1933). Findings from the present study is in line with the previous reports that OG caused distortion and destruction of the architecture and structure of the testicular histology with varying degrees of edema within the interstitial cells in normal mice (Obianime et al., 2010). Reduction in tubular size is associated with detachment and loss of germ cells, which is observed in the testis of rats treated with different drugs (Sasso-Cerri & Miraglia, 2002). Reduction in tubular size is also characterized with structural injury to the Sertoli cells, which disrupts the Sertoli cell-germ cell physical interaction (Richburg & Boekelheide, 1996) and induces programmed cell death among the detached germ cells (Richburg et al., 1999).

Gonadal stem cell damage with low sperm counts or azoospermia, which depends on treatment regimen, route and dose, have been observed with anticancer agents (Lee et al., 2006). A concentration-dependent anti-proliferative activity of OG was documented in prostate cancer (PC-3) cells in-vitro (Ekunwe et al., 2010). Aqueous leaf extract of OG also inhibited proliferation, migration, anchorage independent growth, morphogenesis, induction of COX-2 protein (Nangia-Makker et al., 2007) and matrix metalloproteases (Nangia-Makker et al., 2013) in breast cancer cells. Such tumor prevention potential is common in plants with polyphenolic compounds, anti-oxidants, vitamins, and v-3 fatty acids (Fahey et al., 1997; Pezzuto, 1997; Kobayashi et al., 2000); it is therefore not surprising that polyphenolic and antioxidant components were reported in OG (Venuprasad et al., 2014). Hence, the anticancer activity of OG might have roles to play in its degenerative and deleterious effects on testicular cytoarchitecture and adverse changes in sperm parameters.

Findings from this study showed that although the hypoglycaemic effect of OG is evident with the decreased fructosamine level in diabetic rats; it has debilitating effects on male fertility characterized by reduction in sperm count, increased percentage of abnormal sperm morphology and distortions in testicular cytoarchitecture, which are worsened by diabetes mellitus. Thus, it is important to isolate the active hypoglycaemic component of Ocimum gratissimum to harness it beneficial usage in diabetes mellitus.

**CONFLICT OF INTEREST**

The authors have no conflict of interest to declare.

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| Table 1. Effects of *Ocimum gratissimum* on seminiferous tubular diameter and testicular interstitial space distance in normal and diabetic rats |
|-----------------------------|-----------------------------|
|                            | Seminiferous tubular diameter (µm) | Testicular interstitial space distance (µm) |
| Control                    | 492.79±24.50                 | 34.09±3.80                       |
| OG                         | 137.39±4.62 *                | 66.69±15.86 *                    |
| Dia                        | 153.41±7.43 *                | 58.66±5.23 *                     |
| Dia+OG                     | 172.88±8.53 *                | 56.56±13.97 *                    |

*p<0.05 when compared with the control.*
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