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Research Article

Antioxidant Activity of *Moringa oleifera* Leaf Extract on Testicular Oxidative Stress after Experimentally-induced Cryptorchidism in Male Rats was Optimal at Low Dose

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ABSTRACT

The antioxidant properties of *Moringa oleifera* leaf have been found beneficial in several conditions characterized by oxidative stress. It is widely used for medicinal purposes and its consumption is unregulated. It is also known that standard antioxidant could be prooxidants at doses higher than the recommended. The present study investigated the effects of low and high doses of methanol extract of *Moringa oleifera* leaf (MEMO) on testicular oxidative stress and histology of cryptorchid rats. Thirty-two male Wistar rats were assigned into four groups. Group A was sham-operated and administered distilled water (*p.o*), groups B, C and D were bilaterally cryptorchid and orally treated with distilled water, low dose MEMO (200mg/kg) and high dose MEMO (500mg/kg), respectively. After three weeks of treatments, the left testis was obtained, weighed and assessed for histopathological variation while, superoxide dismutase (SOD) and gamma Glutamyl Transferase (GGT) activities were determined in the right testis. Testicular SOD activity reduced while GGT was increased in the cryptorchid compared with the control. Low dose MEMO-treated cryptorchid had increased SOD with reduced GGT activity compared to the untreated cryptorchid rats. At high dose of MEMO, SOD activity was significantly reduced while GGT activity increased. Testicular histology showed distorted cytoarchitecture and degeneration of germ cells in the cryptorchid rats. This was ameliorated in the low dose MEMO-treated group while there was no difference between the testicular cytoarchitecture of untreated cryptorchid and the high dose MEMO-treated rats. Thus, *Moringa oleifera* leaf extract at low dose ameliorated the disruption of testicular cytoarchitecture while the condition was aggravated by high dose in the experimentally-induced cryptorchid rats.

Keywords: *Cryptorchidism, Moringa oleifera, Superoxide dismutase, Gamma glutamyl transferase.*

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INTRODUCTION

Cryptorchidism or undescended testes is associated with testicular oxidative stress characterized by increased reactive oxygen species (ROS) generation that correlates linearly with germ cell apoptosis (Ahotupa and Huhtaniemi, 1992; Peltola *et al*., 1995). It is thus associated with infertility in the short term and cancer in the long term, with some investigators describing long term cryptorchidism as a precancerous state (Henriksen *et al*., 1995; Sikka, 2001; Agarwal *et al*., 2006). The biochemical response to the excess free radical generation is increased lipid peroxidation. At the cellular level, there is disruption of the germinal epithelium, declined capacity to differentiate normal spermatozoa (Naughton *et al*., 2001) with reductions in the steroidogenic capability of the Leydig cells (Mendis-Handagama *et al*., 1990).

Moringa oleifera, also known as horse-radish tree or drumstick, is widely cultivated in the tropical belt (Jahnn, 1988) of Asia, North and South America and some parts of Europe (Basit *et al*., 2015). Besides the leaves being relatively high in protein content, they are excellent sources of antioxidants (Asma *et al*., 2005) and vitamins A, C and E (Lalas and Tsaknis, 2002). The antioxidant capacity is not only conserved in its leaves but also found to be abundant in its seeds and roots (Jahan *et al*., 2018; Xu *et al*., 2019).

Phytochemical screening and proximate analyses of moringa leaf extracts have demonstrated the presence of a cornucopia of bioactive compounds such as polyphenols, flavonoids, catechins, and tannins. Various extracts of *Moringa oleifera* have been touted as a cure for several ailment ranging from poor eye sight, infertility to diabetes and hypertension. Moreover, due to its high nutritive value, it has also been suggested as a food supplement for regions where under nutrition is prevalent (Anwar *et al*., 2007). Consequently, its use has become widespread leading to the likelihood of adverse reactions arising from overdosage. This scenario is made more likely by the fact that much of the use of moringa products is largely unregulated with no standardization of dosage regimens (Awodele *et al*., 2012).

Functionally, the body has endogenous antioxidant capacity that suppresses overproduction of ROS and protects cellular oxidative stress. This innate antioxidant capacity can be supplemented by exogenous antioxidants such as vitamins A, C, flavonoids and carotenoids commonly found in foods and vegetables (Asma *et al*., 2005). *Moringa oleifera* has been reported to possess antioxidant effects, exerting its protective effects by decreasing liver lipid peroxides and enhancing antioxidants (Ashok and Pari, 2003). It is also well documented that vitamin C (Podmore *et al*., 1998; Kaźmierczak-Barańska *et al*., 2020) and E (Pearson *et al*., 2006; Winterbone *et al*., 2007) at high concentrations could aggravate oxidative stress. It is however not known if *Moringa oleifera* leaf extract with its high concentration of these vitamins could have a dose dependent effect on the amelioration of cryptorchidism-induced testicular oxidative stress. The present study was therefore designed to investigate the antioxidant effect of varied dose of methanol extract of *Moringa oleifera* leaves on experimentally-induced cryptorchidism in male Wistar rats.

MATERIALS AND METHODS

Animals: Thirty-two male Wistar rats (100 - 120g) were used for this study after acclimatizing for two weeks. The animals were housed in plastic mesh cages under standard environmental conditions of 12 hours light and 12 hours dark cycle. The animals were fed with standard pelletized rats feed and water *ad libitum.* Animal handling and experimental protocols were in strict compliance with the NIH publication No 85-23 guidelines on ethical conduct of animal research as updated by the National Research Council (2011).

Preparation of the *Moringa oleifera* Leaf Extract: Fresh leaves of *Moringa oleifera* were obtained in Ogbomosho metropolis. They were identified and authenticated at the Ladode Akintola University of Technology Herbarium under the authority of Professor A. T. J Ogunkunle with assigned Herbarium Voucher Number LHO429. They were shade-dried over a period of six weeks and thereafter ground into powder. Five hundred grams of the powdered leaves were soaked in 250 ml of 70% methanol for 72 hours. The crude concentrated extracts were filtered and extracted by Soxhlet extraction. The extract yield was 30%.

Experimental Procedure: The rats were randomly divided into 4 (A-D) groups of 8 rats each. Group A rats served as the control and were sham-operated (non-cryptorchid). Bilateral cryptorchidism was induced in groups B, C and D followed by treatment with distilled water, 200 mg/kg *Moringa oleifera* and 500 mg/kg *Moringa oleifera*, respectively. Thus, group B served as the Cryptorchid group while Group C and D are the low and high dose *Moringa oleifera* groups. All administrations were done orally for 3 weeks.

Induction of Experimental Cryptorchidism: Cryptorchidism was induced according to the method described by Afolabi *et al* (2013). Briefly, in anaesthetized (50 mg/kg ketamine, *i.p.*) rats, a transverse inguinal incision was made to mobilize the testes into the abdomen through the internal inguinal ring by gentle pressure on the scrotal sac. The gubernacula were cut and the inguinal ring closed preventing movement of the testis back into the scrotum. The incision was then closed with chromic catgut sutures. A topical layer of Streptomycin sulphate (Septomine®, Shrezar Healthcare Nigeria Limited) was applied to the surgical sites to prevent infection. All the rats survived the procedure and subsequently regained consciousness.

Sample Collection: At the end of three weeks treatment, each animal was weighed and euthanized. The testes were removed and weighed immediately. The left testis was rinsed in 1.15% KCl and homogenized for the estimation of antioxidant activities while the right testis was preserved in Bouin's fluid for histopathological assessment. For the homogenization, 0.5g of the left testis was weighed, minced into small pieces and homogenized in 5ml of chilled 10mM Tris/HCl buffer (pH 7.4) and 0.25M sucrose solution. The resulting homogenate was decanted into an eppendorf tube and centrifuged at 3000 revolutions per minute for 10 minutes in a cold centrifuge at 4 ^{0}C. The supernatant was collected into labeled plain sample bottles and preserved at -20 ^{0}C. The supernatant was used to determine gamma glutamyl transferase (GGT) and the superoxide dismutase (SOD).

Assay of Superoxide Dismutase Activity: Superoxide dismutase activities in testicular tissue homogenate was assayed according to Misra and Fridovich (1972)'s method.

Assay of Gamma-Glutamyl Transferase Activity: Testicular gamma-glutamyl transferase (GGT) was assayed using a commercially available kit (Erba kit, Germany) that was based on the method described by Szasz (1969).

Histology of the testis: The right testis was fixed in Bouin's fluid immediately. It was later dehydrated in ascending grade of absolute ethanol then placed in xylene to clear. It was then embedded in paraffin from which a 5µ section was made using a microtone. The section was floated on 20% ethanol at 5 ^{0}C, picked with grease free microscope slide and allowed to drain. It was stained with hematoxylin and eosin then mounted on the slide. Photomicrographs of the sections were made to observe morphological changes across the groups using a microscope at x 400.

Statistical Analysis

The data were presented as Mean ± standard error of mean (SEM). Difference in the mean of the groups was compared with ANOVA and Tukey posthoc test. Mean differences were considered significant at p-value less than 0.05. GraphPad Prism® was used for the statistical analysis.

RESULTS

Effects of *Moringa oleifera* leaf extract on Testicular Weight in Bilaterally induced Cryptorchidism: As shown in figure 1, there was no significant difference (p = 0.52) in the testicular weight of the sham-operated control and the cryptorchid untreated rats. The low dose MEMO treated rats tends to have increased testicular weight while the high dose MEMO treated rats had apparent reduction when compared with the untreated group, however the differences observed in the two treated groups were not statistically significant.

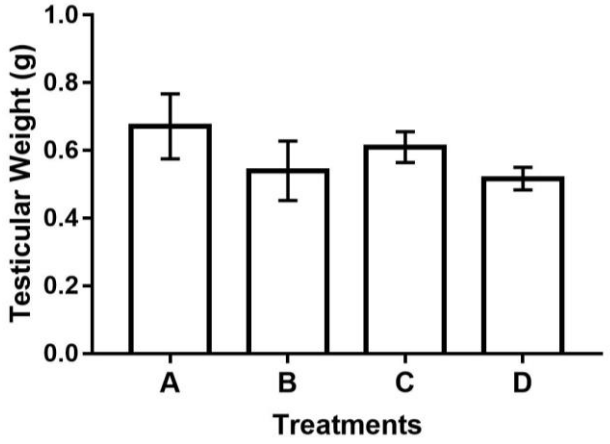

Figure 1.
Effect of methanol extract of *Moringa oleifera* on testicular weight in cryptorchid rats. (A) Control, (B) Cryptorchid, (C) Low Dose Moringa treated Cryptorchid, (D) High Dose Moringa treated Cryptorchid.

Effects of *Moringa oleifera* leaf extract on testicular GGT Activity in Bilaterally induced Cryptorchidism: The activity of GGT in the cryptorchid control was significantly higher than the control (p = 0.02).The low dose treated group had a significantly lowered GGT (p = 0.006) compared to the cryptorchid group while there was no difference in the GGT activity of the high dose treated group and the cryptorchid group (p = 0.08).

Effects of *Moringa oleifera* leaf extract on the testicular SOD Activity in Bilaterally induced Cryptorchidism: Superoxide dismutase activities were significantly reduced in the testes of the cryptorchid rats compared with the control (p = 0.0041). Low dose of *Moringa oleifera* treatment caused significant increased SOD activity compared with the cryptorchid rats (p = 0.00052) while at high dose, *Moringa oleifera* treatment further lowered SOD activity when SOD activity when compared with the cryptorchid group (Figure 3).

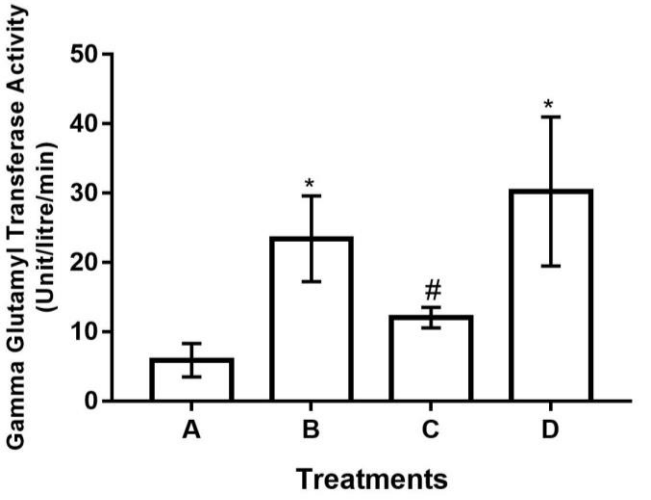

Figure 2.
Effect of ethanol extract of *Moringa oleifera* on gamma glutamyl transferase activities in the testes of cryptorchid rats. (A) Control, (B) Cryptorchid, (C) Low Dose Moringa treated Cryptorchid, (D) High Dose Moringa treated Cryptorchid. $^{*}P < 0.05$ compared with the control, $^{\#}P < 0.05$ compared with the cryptorchid rats

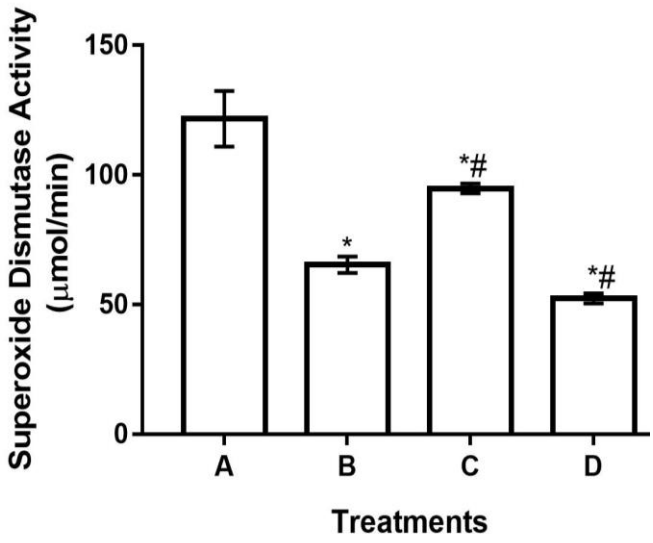

Figure 3.
Effect of methanol extract of *Moringa oleifera* on superoxide dismutase activities in the testes of cryptorchid rats. (A) Control, (B) Cryptorchid, (C) Low Dose Moringa treated Cryptorchid, (D) High Dose Moringa treated Cryptorchid. $^{*}P < 0.05$ compared with the control, $^{\#}P < 0.05$ compared with the cryptorchid rats.

Effects of *Moringa oleifera* leaf extract on Testicular Cytoarchitecture in Bilaterally induced Cryptorchidism: The histology of the control testes showed normal lining of the seminiferous tubule lamina propria by Sertoli cells embedded with spermatogonia. The lumens were filled with strands of spermatozoa, Plate 1A. By contrast, the cryptorchid (Plate 1B) showed widened lumen of the seminiferous tubules which were virtually devoid of strands of spermatozoa. There was evidences of degenerative changes in the seminiferous epithelium characterized by indistinct sertoli cells with severe loss of germ cells.

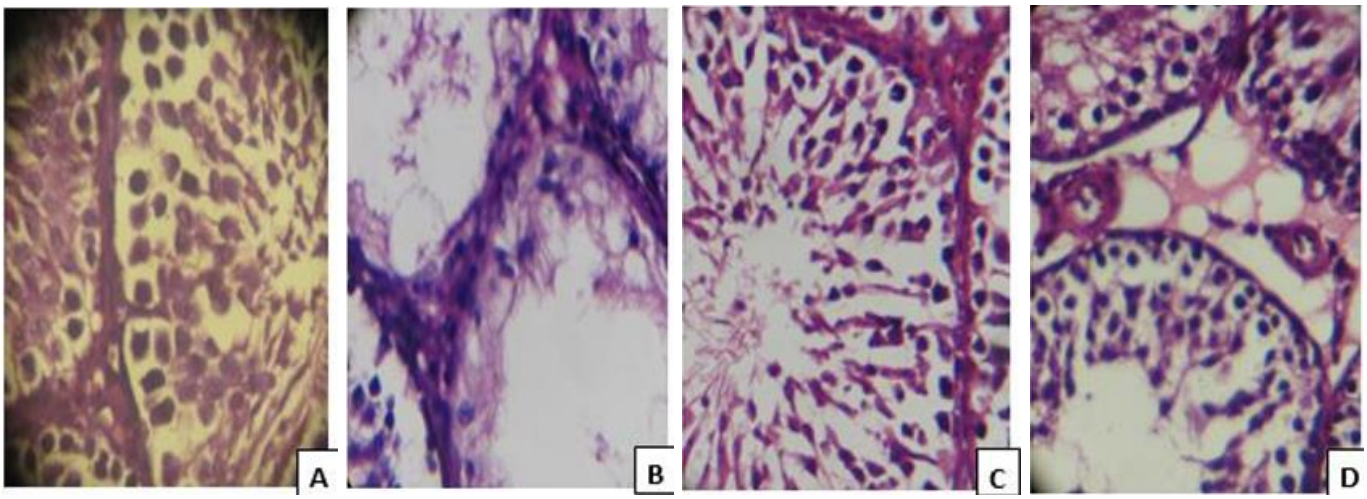

Figure 4.
Photomicrograph showing the effect of methanol extract of *Moringa oleifera* on testicular cytoarchitecture of cryptorchid rats. (A) Control, (B) Cryptorchid, (C) Low Dose Moringa treated Cryptorchid, (D) High Dose Moringa treated Cryptorchid. H & E, X400. Note abnormal seminiferous tubules with severe loss of germ cells, sloughed sertoli cells and widened lumen devoid of spermatozoa in Cryptorchid (B) and High Dose Moringa treated Cryptorchid(D).

Testes of the low dose moringa treated group (Plate 1C) was almost indistinguishable from the sham operated group showing largely normal seminiferous tubules with the lamina propria lined by spermatogonia embedded in Sertoli cells. At high dose of *Moringa oleifera* (Plate 1D), attenuation of the seminiferous epithelium and interstitium with mild degenerative changes characterized by very few tubules with normal germ cell layers were visible.

DISCUSSION

Excessive generation of free radicals has been shown to be the causative factor for cellular disruptions observed in cryptorchidism. In rat models, experimental cryptorchidism caused rapid degeneration of testicular germ cells, increased rate of apoptosis, malignant cell transformation and infertility (Billing and Hsueh, 1994; Davis and Firlit, 1996) which have been linked to increased oxidative stress. Elevation of testicular GGT activity and concomitant reduction in SOD activity observed in the cryptorchid rats of this study are indicators of oxidative stress. These findings agree with the report of earlier studies on depleted antioxidant capacity (Radi *et al*., 1991) and elevated GGT level in the cryptorchid testes as invariable consequence of oxidative stress during cryptorchidism (Afolabi *et al*., 2009).

The administration of low dose moringa extract to cryptorchid rats resulted in increased SOD and reduced GGT concentration in testicular homogenates compared to the untreated cryptorchid control which had reduced SOD and increased GGT activity which indicated a reduction in the oxidative stress. This is in line with the well documented antioxidant activity of *Moringa oleifera* (Asma *et al*., 2005; Jahan *et al*., 2018; Xu *et al*., 2019). The rich vitamin C and E content of *Moringa oleifera* (Lalas and Tsaknis, 2002) seems to have roles to play in the observed reduced oxidative stress in the low dose MEMO treated rats of the current study. It has been previously reported in other studies that the antioxidant properties of vitamin E is associated with increased activation of SOD and decreased activation of NADPH oxidase and xanthine oxidase enzyme (Chen *et al*., 2001). Xanthine oxidase has been reported to contribute largely to the testicular free radical pool (Yaman *et al*., 1999). Given that various reports have shown that 100g moringa leaves contain more than 200mg of vitamin C and 115mg of vitamin E (Fuglie, 2000), it is quite possible that the synergistic action of vitamins C and E is responsible in part for the observed effects on the antioxidant and oxidative stress biomarkers in the testes.

It is worthy to note that, high dose moringa extract treated rats did not show any evidence of reduction in oxidative stress nor its testicular effects. Their testicular SOD activity was not different from the untreated cryptorchid control neither was there any appreciable reduction of GGT activity following treatment with high dose moringa. Histological findings also showed no improvement in the changes brought about by experimental cryptorchidism. These findings were rather unexpected as other studies using different doses showed dose dependent effect with the higher doses showing greater effect in reducing oxidative stress and its effects (Afolabi *et al*.,2013). Since *Moringa oleifera* leaves are known to have high concentrations of vitamins C and E, at high dose of moringa leaf extract the concentration of vitamins C and E may also be high. Studies have shown that high concentration of vitamin C suppresses the activity of superoxide free radicals intracellularly (Rietjens *et al*., 2002) and low doses of vitamin E have been found also to be more effective than the high doses (Miller *et al*., 2005; Hathcook, 2014). These studies taken together may indicate that there is an optimal combination of the two vitamins that have beneficial effect and that the optimal combination is achieved at the lower dose (200mg/Kg body weight) of the extract. At the higher dose (500mg/Kg body weight) of the extract, the optimal combination necessary for the synergistic action of vitamins C and E to occur may have been exceeded. Indeed, an earlier study has shown that combining Vitamins C and E did not reverse the oxidative stress of cryptorchidism while vitamins

C and E given separately did reverse the effects of cryptorchidism in rats (Afolabi *et al*., 2012). Furthermore, reports have shown that high dose of vitamin E may be pro-oxidant and could cause oxidative damage to the cells (Brown and Crowey, 2005). High concentration of vitamin E in high dose moringa treated may therefore be responsible for the increased GGT concentration and reduced SOD concentration on treatment with high dose moringa.

The lack of effect of the high dose moringa observed in this study lend credence to the reports of Oyagbemi *et al*. (2013) and Omobowale *et al*. (2014) that methanolic extract of *Moringa oleifera* at 400 mg/kg and above may predispose hepatic and kidney damage when consumed chronically. In fact, Kim *et al* (2018) posited that the effective dose at which *Moringa oleifera* could have no adverse effect is 257 mg/kg/day. We therefore conclude that high dose of MEMO had no beneficial effect on bilaterally cryptorchidism-induced oxidative stress and may indeed aggravate the oxidative stress and its deleterious effect on testicular cytoarchitecture. Further studies to determine the optimal beneficial dose of *Moringa oleifera* is imperative.

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