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EFFECTS OF METHANOLIC EXTRACT OF *MORINGA OLEIFERA* LEAVES ON SEMEN AND BIOCHEMICAL PARAMETERS IN CRYPTORCHID RATS

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Abstract

While anti-oxidant effects of *Moringa oleifera* in much oxidative stress related diseases have been well reported, cryptorchidism on the other hand has been shown to cause oxidative stress. However, study is scanty on the likely role of *Moringa oleifera* in reducing cryptorchidism-induced oxidative stress in rats has not been studied. The present study looked into the effects of methanolic extract of *Moringa oleifera* leaves (MEMO) on semen and biochemical parameters in cryptorchid rats. Twenty male albino rats (200-250g) were randomly divided into 4 groups (n=5 each). Groups A and B were sham-operated and treated with corn-oil and 200mg/kg of MEMO respectively, while groups C and D were rendered cryptorchid and also treated with corn-oil and 200mg/kg of MEMO respectively. Cryptorchid rats had lower testicular weight, sperm count, germ cell count, testicular superoxide dismutase (SOD) concentration, testicular total protein and higher testicular malondialdehyde (MDA) concentration compared to sham-operated rats. MEMO had no significant effect on testicular weight and MDA concentration, while it significantly increased sperm count, germ cell count, testicular SOD and total protein in the cryptorchid rats. The present study suggests that MEMO ameliorates cryptorchidism associated germ cell loss and oxidative stress.

Keywords: Antioxidants, Cryptorchidism, *Moringa oleifera*, Oxidative stress, Sperm count, Germ cell count

Introduction

Cryptorchidism results from the failure of the testis to descend from the abdominal region into the scrotal sac. It occurs naturally (Lim et al., 2001), and can also be induced experimentally (Ahotupa and Huhtaniemi, 1992). It is the most significant risk factor for testicular cancer, increasing the risk 2.5-11 fold (Benson et al., 1991). Its aetiology is for the most part unknown and appears to be multifactorial (Krausz et al., 2000). In most mammals, the testis is kept between 3-5^{0}C below body temperature. A slight increase in temperature for a short or long period results in a rapid loss of mature germ cells. The increased testicular temperature in cryptorchidism has long been associated with increased testicular oxidative stress (Ahotupa and Huhtaniemi, 1992; Misro et al., 2005; Peltola et al., 1995). Moreover, cryptorchidism has also been shown to induce an increase in ROS, which correlated with increased germ cell apoptosis and alterations in the expression of a number of genes associated with energy and lipid metabolism, stress response, and redox reactions (Li et al. 2006). Testicular tissue under increased temperature in vitro also showed an increased susceptibility to oxidative stress and germ cell apoptosis (Ikeda et al., 1999). The increase in ROS during cryptorchidism has also been correlated with a decline in testosterone (Chaki et al., 2005), and oxidative stress.

Oxidative stress results from the production of oxygen radicals in excess of the antioxidant capacity of the stressed tissue. Any oxidising radical is a potential agent of oxidative stress. The major antioxidant enzymes in mammals are Superoxide dimutase (SOD), Catalase and Glutathione peroxidase, which are all endogenous. Substances like vitamins A, C, flavonoids and carotenoids are examples of exogenous antioxidants found in food (Asma et al., 2005).

Moringa oleifera (Horse-radish tree or Drumstick) is a medium-sized (about 10 metres high) tree belonging to, and most widely known species of the Moringaceae family cultivated in the tropical belt (Jahn, 1988). The leaves and the pods are known to have a high content of protein, minerals and vitamins (Council of Scientific and Industrial Research, 1962).

Moringa oleifera leaves (Asma et al., 2005) and seeds (Lalas and Tsaknis, 2002) are excellent sources of antioxidants. Its extract exerts protective effects by decreasing liver lipid peroxides and enhancing antioxidants (Ashok and Pari, 2003).

Moringa preparations have been cited in the scientific literatures as having antibiotic, antitrypanosomal, hypotensive, antispasmodic, antiulcer, anti-inflammatory, hypo-cholesterolemic, and hypoglycemic activities, as well as having considerable efficacy in water purification by flocculation, sedimentation, antibiosis and even reduction of Schistosome cercariae titre (Fahey, 2005).

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In tissues like testis with high rate of metabolism and cell replication, oxidative stress can be especially damaging, which makes the antioxidant capacity of the tissues very important. Testicular oxidative stress appears to be a common feature in much of what underlies male infertility (Canto et al., 2003), which suggests that there may be benefits to developing better and cheaper antioxidant therapies for relevant cases of hypospermatogenesis. Therefore, the present study was designed to investigate the antioxidant activity of the methanolic extract of *Moringa oleifera* leaves in an experimentally-induced cryptorchidism in rats.

Methods

Animals

Twenty male albino rats (200-250g) were purchased from the Institute of Medical Research and training, University of Ibadan College Hospital, Nigeria and were acclimated to their new environment. They were fed with standard laboratory pellet diet (Bova Jay Feeds Nig. Ltd, Ogbomoso) and water ad libitum. The rats were kept under condition of uniform humidity and temperature on a 12-h light-dark cycle. The rules guiding animal experimentation and humane treatment of animals used in biomedical research were observed.

Selection of Moringa oleifera and preparation of methanol extract

Fresh leaves of *Moringa oleifera* were obtained from the National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Nigeria and were identified and authenticated at the Botany section of the Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Nigeria. They were shade-dried over a two week period and thereafter reduced to powder form. 630g of the powdered leaves were obtained and exhaustively extracted with 250ml of 70% methanol for 72 hours. The mixture was preserved in a brown bottle and shaken everyday to ensure proper diffusion and circulation. The crude concentrated extracts were filtered and exposed in the absence of sun for the methanol to dry off and also for the extract to solidify for easy measurement.

Experimental procedure

All necessary protocols were followed to ensure the humane treatment of the animals. Twenty rats were randomly divided into 4 groups (A-D), consisting of 5 rats per group: Group A Rats were pre-treated with corn-oil (vehicle) for 2 weeks, sham-operated (non-cryptorchid) on the 15th day and post-treated with corn-oil for another 2 weeks. Group B Rats were pre-treated with 200mg/kg of methanolic extract of *Moringa oleifera* leaves (MEMO) for 2 weeks, sham-operated (non-cryptorchid) on the 15th day and post-treated with 200mg/kg of MEMO for another 2 weeks. Group C was pre-treated with corn-oil for 2 weeks, rendered cryptorchid on the 15th day and post-treated with corn-oil for another 2 weeks. Group D was pre-treated with 200mg/kg of MEMO for 2 weeks, rendered cryptorchid on the 15th day and post-treated with MEMO for another 2 weeks. Cryptorchidism was induced as previously described (Afolabi et al., 2009).

Sample collection

At the end of 15 days post-operation, the rats were sacrificed by cervical dislocation. The testes were removed and weighed immediately. The caudal end of the epididymis was cut for sperm analysis. One of the testes was washed with "washing buffer" and then homogenised for the estimation of antioxidant activities and the other testis was preserved for histological analysis in a 10% formalin solution.

Semen analysis

Semen was collected from the caudal end of the epididymis after sacrificing the animals and precautions were taken to avoid exposure to heat. To examine sperm count, 0.1ml sample of semen was diluted with 1% formalin in 2ml volumetric flask. The flask was shaken very well and the sample was placed in a counting chamber (haemocytometer). After the sperms had settled on the grid, they were counted in the five squares using objective lens 40 after the semen has been diluted 1:10. The number of sperms in five squares is multiplied by 10^6 to determine the number of sperms per millilitre.

Histological processing of testicular tissue

The testicular tissues were prepared for histological preparation as described elsewhere (Afolabi et al., 2009).

Determination of superoxide dismutase (SOD)

Superoxide dismutase activity was assayed spectrophotometrically by inhibition of epinephrine autoxidation as previously described (Misra and Fridovish, 1972).

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Determination of Malondialdehyde (MDA)

Malondialdehyde activity was assayed spectrophotometrically as previously described (Ohkawa *et al., 1979*).

Total Protein measurement

Testicular total protein was measured as previously described (Koller, 1984).

LD50 experiment

The toxicity of the extract was also studied by LD50 experiment. Two groups of six male rats weighing about (200-250g) were orally administered 2g/kg or3g/kg of MEMO. Rats were then observed continuously for their gross behavioural, neurological, autonomic and toxic effects up to 24 h. Food consumption, faeces and urine were also examined at 2 h and then at 6 h intervals for 24 h.

Data processing

Data were analysed using Microsoft Excel statistical package. All values given are the mean±S.D. of the variables measured. Significance was assessed by the Student's T-test. P-Values of 0.05 or less were taken as statistically significant.

Results

Effects of Moringa olifera on the testicular weight in non-cryptorchid and cryptorchid rats

In the non-cryptorchid (sham-operated) group, there was no significant difference between the testicular weight in the rats treated with vehicle and those treated with MEMO ($P>0.05$). Testicular weight was significantly lower ($P<0.001$) in the cryptorchid rats than in the non-cryptorchid rats treated with vehicle, that is, cryptorchid rats had lower testicular weight. There was no significant difference ($P>0.05$) in the testicular weight in the cryptorchid rats treated with either MEMO or vehicle; that is, MEMO had no effect on the testicular weight of the cryptorchid rats (Table 1).

Effects of Moringa oleifera on the sperm count in non-cryptorchid and cryptorchid rats

In the non-cryptorchid group, there was no significant difference between the sperm count in the rats treated with vehicle and those treated with MEMO ($P>0.05$). Sperm count was significantly lower ($P<0.001$) in the cryptorchid rats than in the non-cryptorchid rats treated with vehicle, that is, cryptorchid rats had lower sperm count. Sperm count was significantly higher ($P<0.001$) in the cryptorchid rats treated with MEMO than in those treated with vehicle. Moreover, there was no significant difference ($p>0.05$) between the sperm count of the cryptorchid rats treated with MEMO and non-cryptorchid rats treated with vehicle. These showed that MEMO significantly increased the sperm count in the cryptorchid rats (Table 1).

Effects of Moringa oleifera on the germ cell count in non-cryptorchid and cryptorchid rats

In the non-cryptorchid group, there was no significant difference between the germ cell count in the rats treated with vehicle and those treated with MEMO ($P>0.05$). Germ cell count was significantly lower ($P<0.001$) in the cryptorchid rats than in the non-cryptorchid rats treated with vehicle, that is, cryptorchid rats had lower germ cell count. Germ cell count in the cryptorchid rats treated with MEMO, though significantly lower ($p<0.05$) than in non-cryptorchid rats treated with vehicle, was significantly higher ($P<0.05$) than in the cryptorchid rats treated with vehicle. These showed that MEMO significantly increased the germ cell count in the cryptorchid rats (Table 1).

Effects of Moringa olifera on the testicular SOD concentration in non-cryptorchid and cryptorchid rats

In the non-cryptorchid group, there was no significant difference between the testicular SOD concentration in the rats treated with vehicle and those treated with MEMO ($P>0.05$). Testicular SOD concentration was significantly lower ($P<0.05$) in the cryptorchid rats than in the non-cryptorchid rats treated with vehicle, that is, cryptorchid rats had lower testicular SOD concentration. Testicular SOD concentration in the cryptorchid rats treated with MEMO, apart from not being significantly different ($p>0.05$) from the non-cryptorchid rats treated with vehicle, was significantly higher ($P<0.05$) than in those treated with vehicle. These showed that MEMO significantly increased the testicular SOD concentration in the cryptorchid rats (Table 2).

Effects of Moringa olifera on the testicular MDA concentration in non-cryptorchid and cryptorchid rats

In the non-cryptorchid group, there was no significant difference between the testicular MDA concentration in the rats treated with vehicle and those treated with MEMO ($P>0.05$). Testicular MDA concentration was significantly higher ($P<0.05$) in the cryptorchid rats than in the non-cryptorchid rats treated with vehicle, that is, cryptorchid rats had higher

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testicular MDA concentration. However, there was no significant difference ($p>0.05$) between the testicular MDA concentration in the vehicle and MEMO treated cryptorchid rats (Table 2).

Effects of Moringa olifera on the testicular total protein concentration in non-cryptorchid and cryptorchid rats

In the non-cryptorchid rats, testicular concentration of total protein was significantly higher in the MEMO treated than in vehicle treated rats ($P<0.05$). Testicular total protein concentration was significantly lower ($P<0.01$) in the cryptorchid rats than in the non-cryptorchid rats treated with vehicle, that is, cryptorchid rats had lower testicular total protein concentration. Testicular total protein concentration in the cryptorchid rats treated with MEMO, in addition to being significantly higher ($p<0.01$) than the non-cryptorchid rats treated with vehicle, was significantly higher ($P<0.001$) than in the cryptorchid rats treated with vehicle; that is, MEMO significantly increased the testicular total protein concentration in the cryptorchid rats (Table 2).

Table 1: Effects of methanolic extract of *Moringa oleifera* leaf (MEMO) (200mg/kg) on testicular weight, sperm count and germ cell count in non-cryptorchid and cryptorchid rats. Values are expressed as Mean±SD. (N=5)

	A (Sham operated, Corn-oil treated)	B (Sham-Operated, MEMO treated)	C (Cryptorchid, corn-oil treated)	D (Cryptorchid, MEMO treated)
Testicular weight (g)	1.98 (±0.09)	2.08 (±0.03)	0.75 (±0.09)***	0.89 (±0.04)***
Sperm count (x10^6/ml)	47.08 (±1.39)	49.44 (±2.03)	3.04 (±1.57)***	44.06 (±1.74)$^{###}$
Germ cell count (x10^6)	202.2 (±32.02)	193.8 (±16.78)	25.5 (±3.82)***	96.4 (±25.06)$^{*, \#}$

$^{(*, ***)}$ signify $p<0.05$, $p<0.001$ respectively vs. group A rats. $^{(\#, ###)}$ signify $p<0.05$, $p<0.001$ respectively vs. group C rats

Table 2: Effects of methanolic extract of *Moringa oleifera* leaf (MEMO) (200mg/kg) on the testicular Superoxide dismutase (SOD), Malondealdehyde (MDA) and Total protein (TP) concentration in non-cryptorchid and cryptorchid rats. Values are expressed as Mean±SD. (N=5)

	A (Sham operated, Corn-oil treated)	B(Sham-Operated, MEMO treated)	C(Cryptorchid, corn-oil treated)	D(Cryptorchid, MEMO treated)
SOD (u/mg Protein)	1.04 (±0.20)	1.28 (±0.15)	0.56 (±0.1)*	0.96 (±0.1)$^{\#}$
MDA (X10^{-4}) (µmol/mg Protein)	40.1 (±3.69)	37.8 (±1.21)	53.2 (±4.39)*	45.6 (±0.32)
TP (mg/ml)	0.99 (±0.03)	1.19 (±0.06)*	0.77 (±0.04)**	1.22 (±0.05)$^{**, ###}$

$^{(*, **)}$ signify $p<0.05$, $p<0.01$ respectively vs. group A rats, while $^{(\#, ###)}$ signify $p<0.05$, $p<0.001$ respectively vs. group C rats

Discussion

The observed reduction in testicular weight in the cryptorchid rats in the present study is similar to the previous reports of Nef and Parada (1999) and Zimmermann (1999). Declined intratesticular testosterone, elevated temperature and high oxidative stress following cryptorchidism probably affect cell viability and trigger a fast pace cell removal through giant cell formation (Chaki et al., 2005). The reduction in testicular weight in cryptorchids has been attributed to the loss of spermatocytes and spermatids. The present study also shows that methanolic extract of *Moringa oleifera* (MEMO) had no effect on testicular weight in both cryptorchid and non-cryptorchid rats. This result shows that MEMO is not effective in reversing the cryptorchidism-induced decrease in testicular weight.

The lack of effect of MEMO on the sperm count in the non-cryptorchid rats may be because of little or no oxidative stress in these rats. The observed reduction in sperm count in cryptorchid rats in the present study is similar to the previous reports in rat (Afolabi et al., 2009; Ahotupa and Huhtaniemi, 1992; Duru et al., 2011; Oyewopo and Togun, 2005), mice (Nef and Parada, 1999; Zimmermann, 1999), and human (Moretti et al., 2007). This may be a result of the impairment of spermatogenesis associated with cryptorchidism (Ahotupa and Huhtaniemi, 1992; Clegg, 1963; Dada et al., 2002; Dohle et al., 2005; Moore, 1924). Moreover, the impairment of spermatogenesis has been reported to be more severe in patients with bilateral cryptorchidism compared with unilateral cryptorchidism or retractile testes (Caroppo et al., 2005). Oxidative stress is a mediator of sperm cells dysfunction (Aitken and Clarkson, 1987). Excessive production of ROS results in destruction of the antioxidant capacity of spermatozoa and seminal plasma, causing oxidative stress which damages spermatozoa membrane and causes infertility. Treatment with MEMO increased the sperm count of the cryptorchid rat. This suggests that MEMO potently reverses the reduction in sperm count induced by cryptorchidism, probably by boosting the antioxidant level.

The lack of effect of MEMO on the germ cell count in the non-cryptorchid rats in the present study may also be a result of little or no oxidative stress in these rats. A slight increase in testicular temperature for a short time or an experimental cryptorchidism which is more convenient for longer periods results in rapid loss of mature germ cell. Moreover, surgical induction of cryptorchidism in experimental animals causes rapid degeneration of testicular germ cells (Afolabi et

al., 2009; Davis and Firlit, 1966t; Nef and Parada, 1999; Zimmermann, 1999). The present study also showed that cryptorchidism causes reduction in germ cells. This result may be a product of cryptorchidism-induced oxidative stress, leading to increased germ cell destruction. The present study further observed that MEMO increased the germ cell count in the cryptorchid rats probably by reversing the cryptorchidism-induced oxidative stress as a result of its antioxidant property.

The increase in SOD level in the non-cryptorchid rats pre-treated with MEMO than those treated with control showed that MEMO has an anti-oxidant effect in these rats. Superoxide dismutase (SOD) has been reported as one of the most important enzymes in the enzymatic antioxidant defence system. It scavenges the superoxide anion to form hydrogen peroxide and thus diminishing the toxic effect caused by the former. The observed reduction in the plasma SOD level in the cryptorchid rats than in the non-cryptorchid rats in the present study is consistent with the current report of Duru et al. (2011). This finding may be a result of the testicular damage which results from cryptorchidism, leading to excessive generation of free radicals, or direct effect of the elevated temperature in the abdominal testis (Ahotupa and Huhtaniemi, 1992). The observed increase in the plasma SOD level in the cryptorchid rats treated with MEMO than in those treated with control provided evidence for the effectiveness of MEMO in reversing cryptorchidism-induced oxidative stress.

The lack of effect of MEMO on the plasma MDA level in the non-cryptorchid rats in the present study may also be a result of little or no oxidative stress in these rats. Previous study has shown that testicular tissues under increased temperature as in cryptorchidism have an increased susceptibility to oxidative stress, causing an increased level of lipid peroxidation (Duru et al., 2011; Ikeda et al., 1999; Peltola et al., 1995). The present study also showed that MDA production is elevated in the cryptorchid rats, leading to lipid peroxidation, tissue damage and failure of antioxidant defence mechanism to prevent formation of excessive free radicals. The lack of effect of MEMO in the plasma MDA level of cryptorchid rats showed that MEMO is not effective in reversing the cryptorchidism-induced increase in lipid peroxidation in rats.

The observed reduction in the total protein level in the cryptorchid rats compared to the non-cryptorchid rats treated with vehicle showed that cryptorchidism causes reduction in total protein level. This finding may be a result of accelerated mRNA degradation in cryptorchid rats, resulting in reduced half-life of protein mRNA (Legare et al., 2004). Treatment with MEMO increased the total protein level in the cryptorchid rats. This result may be a product of its previously reported cytoprotective role, preventing irreversible damage to cellular proteins by binding to unfolded or partially malfolded peptides to retard thermal denaturation and aggregation of cellular proteins (Morimoto et al., 1994). This role is observed in the comparison of the testicular level of total protein in the cryptorchid extract treated rats and the sham-operated extract treated rats, which shows no significant difference.

The present study suggests that *MEMO* ameliorates cryptorchidism-associated germ cell loss and oxidative stress.

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