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EVALUATION OF THE PROTECTIVE ROLE OF AQUEOUS EXTRACT OF MORINGA OLEIFERA LEAF AGAINST OXIDATIVE STRESS AND HISTOLOGICAL ALTERATIONS IN TESTES OF RATS UNDER INSULIN RESISTANT CONDITION

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ABSTRACT

Objective: To investigate the effect of aqueous extract of Moringa oleifera (AEMO) leaf on the oxidative stress, antioxidant status as well as histo pathological changes in testes of rats under insulin resistant condition. Methods: The male Wistar rats were randomly divided into four groups of six rats each : C (Control), C+MO (control rats administered with AEMO), F (fructose fed rats) and F+MO (fructose fed rats administered with AEMO), Insulin resistance was induced by high fructose diet. AEMO was administered orally at a dose of 200 mg/kg body weight for 60 days. Relative organ weight was calculated. Phytochemical screening of AEMO was done by standard methods. Oxidative stress and the status of antioxidant enzymes were estimated in testes and the histological changes in testes were studied. Statistical significance of the results was evaluated by Duncan's Multiple Range Test. Results: Phytochemical analysis showed the presence of pharmacologically important phytochemicals. The reduced relative weight of the testes was observed in F group. F group showed increased lipid peroxidation, decreased levels of reduced glutathione, reduced activities of glutathione reductase, glutathione S transferase, superoxide dismutase, catalase and increased activity of Sorbitoldehydrogenase. Histological pictures of testes of F group showed pathological changes. However, administration of AEMO corrected the alterations towards normalcy Conclusion: In this study, AEMO ameliorated oxidative stress, exhibited antioxidant potential and played a role in the protection of testicular structural integrity under insulin resistant condition.

Key words: Moringa oleifera, Insulin resistance, Testes, antioxidants, oxidative stress, Histopathology

INTRODUCTION

High dosage of fructose in the diet has been shown to induce insulin resistance, hyperinsulinaemia, hyperglycemia, glucose intolerance and hyperlipidemia in Wistar rats [1]. Most of the metabolic effects of fructose are due to its rapid utilization by the liver and its entry into the pathway of glycolysis or gluconeogenesis at the triose phosphate levels after bypassing the phosphofructokinase regulatory step [2] leading to a far reaching consequences to carbohydrate and lipid metabolism. Glucose, produced as a result of gluconeogenic precursors from fructose metabolism, stimulates insulin release, but the fructose-induced insulin resistance prevents the insulin from effectively metabolizing glucose. Insulin resistance is characterized by impaired glucose tolerance, which ultimately leads to type 2 diabetes.

During diabetes, persistent hyperglycemia causes increased production of free radicals especially reactive oxygen species (ROS), in all tissues from glucose auto-oxidation and protein glycation [3]. In addition, hyperinsulinemia in insulin resistant condition and enhanced free fatty acids (FFA) observed in diabetes and insulin resistant condition are also sources of free radicals [4], [5]. Over production of ROS causes oxidative stress, which is currently suggested as the mechanism underlying diabetic complications. Normalizing ROS generation showed to prevent the long-term complications of diabetes [6].

Recent years have witnessed a renewed interest in plants as pharmaceuticals. Many studies have shown that the secondary metabolites produced by plants possess antioxidant property, which play a major role against functional and cellular damage caused by ROS. Several phytochemicals were reported to act against the

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deleterious effects of oxidative stress such as anthraquinones of aloe vegetable [7], saponins from Pinax ginseng [8], polyphenols [9] and flavonoids from Sideritis raeseri [10] and the active tannoid principle isolated from Emblica officinalis [11].

In the present study the efficacy of aqueous extract of Moringa oleifera (AEMO) leaf in combating the oxidative stress in testes of insulin resistant rats was investigated. Moringa oleifera Lam (syn Pterigosperma Geartn) belongs to the monogeneric family Moringaceae and it is one of the best known, most widely distributed and naturalized species [12]. It is popularly known as drumstick or horseradish. Moringa oleifera was well known to the ancient world, but only recently has it been 'rediscovered' as the "Miracle tree" with a tremendous variety of potential uses. Leaves, immature pods, flowers and fruits of this plant are edible and are highly nutritive. Moringa leaves have been reported to be a rich source of β-carotene, protein, Vitamin C, calcium, potassium and essential amino acids which make them an ideal source of dietary supplement [13].

M. oleifera contains nitrile mustard oil glycosides and thiocarbamate glycosides which are antihypertensive [14]. Niazinin, Niazimicin and Niazimin A+B were isolated from Moringa leaves which were found to control blood pressure [15]. Our earlier studies of AEMO for trace elements by Particle Induced X-ray Emission (PIXE) technique revealed the presence of many physiologically and biochemically important trace elements: Ca, Zn, K, Mn, Na, Sr, Fe,Ni, Cu [16].

Moringa leaves were found to possess many medicinal uses. The leaves possess strong antioxidants and radical scavenging activities and enhance the process of spermatogenesis in mice [17], have shown to correct hyperglycemia, hyperlipidemia in both type 1 and type 2 diabetic rat models [1], inhibit the growth of pathogenic microorganisms [18], reported to be useful in treating hyperthyroidism [19].

As a result of scientific evidence, M. oleifera is coming to the forefront as an important source of naturally occurring phytochemicals.

MATERIALS AND METHODS

Chemicals

Thiobarbituric acid, pyrogallol were obtained from Sigma Chemical Co., St Louis, MO, USA. All other chemicals and solvents were procured from Sisco Research Laboratories (p) Ltd., Mumbai. India.

Plant material

Aqueous extract of Moringa oleifera leaf (AEMO) powder (Product Code P/DSM/MOOL-01, Batch Number P8060947) was purchased from Chemiloids (manufactures and exporters of herbal extracts, Vijayawada, Andhra Pradesh, India).

Qualitative screening for phytochemicals.

The extract was qualitatively tested for the presence of Alkaloids, flavonoids, gallic tannins, catecholic compounds, phenols, saponins,and triterpines by following the procedures of Brain and Tunfer [20]; Sofowora [21]; Trease and Evans [22].

Experimental Design

In the present study a total of 24 rats (12 normal rats and 12 high fructose fed rats) were randomly divided into four groups of six rats each : C (Control), C+MO (control rats administered with AEMO), F (fructose fed rats), F+MO (fructose fed rats administered with AEMO). AEMO was administered by orogastric tube at a dose of 200 mg/kg body weight for 60 days. At the end of the experimental period the rats were sacrificed by cervical decapitation and testes were immediately dissected out and weighed.

Animals

Male albino Wistar rats of age 4-5 weeks with a body weight of 150-160 g were procured and acclimatized for 7 days to animal house maintained at a temperature of 22 ± 2º C. The study was approved by Animal Ethics Committee of S.K.University, Anantapur (Reg. no. 470/01/a/CPCSEA). The animal room was regulated by a 12/12 h light/ dark schedule. Two animals were housed per cage. All rats were fed on a standard pellet diet and water before dietary manipulation.

Induction of Insulin Resistance

Insulin resistance (IR) was induced by feeding the rats the fructose-enriched diet (66% fructose, 18% protein, 8% fat, 4% cellulose, 3% mineral and 1% vitamin mix) throughout the experimental period of 60 days.

Fructose enriched diet

Fructose diet was procured from National Centre for Animal Science, National Institute of Nutrition (Hyderabad, India).

Oxidative stress markers and Antioxidant Enzymes

A 10% tissue homogenate was prepared in 0.15 M KCl using pestle and mortar at 4°C. The extent of lipid peroxidation (LPO) was determined by assaying malondialdehyde (MDA) formation according to method of Utley et al. [23]. Total reduced glutathione (GSH) content was measured following the method of Ellman's [24].

The protein content of the homogenate was estimated by the method of Lowry et al [25].

A portion of the homogenate was centrifuged at 4°C in Eppendorf centrifuge at 12,000 rpm for 45 minutes. The clear supernatant was used for the assay of Glutathione reductase (GR; E.C 1.6.4.2); Pinto and Bartley [26], Glutathione-S-transferase (GST; E.C 2.5.1.18) Habig et al. [27], Catalase (CAT; E.C 1.11.1.6); Beers and Sizer [28], Superoxide dismutase (SOD; E.C 1.15.1.1); Soon and Tan [29] and Sorbitol dehydrogenase (SDH; E.C 1.1.1.14); Asada and Galambos [30].

Relative testicular weight and Histological study

The relative weight of testes was calculated from body weight and absolute testes weight.

Immediately after separation, the testes were weighed and fixed in 10% formalin and later were embedded in paraffin. Sections of 3 microns thickness were cut from tissue blocks by microtome. Using a heated tissue separator (water bath), the sections were uniformly separated on a glass slide and drained. Harris' Hematoxylin and Eosin stain was used for staining the tissue sections following the procedure from the Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology [31].

Statistical Analysis

The results were expressed as means ±S.E.M. Data were analysed for significant differences using Duncan's Multiple Range (DMR) test ($p<0.05$) Duncan [32].

RESULTS

The phytochemical screening of AEMO revealed the presence of alkaloids, flavonoids, gallic tannins, phenols, saponins and catecholic compounds and steroids indicating the presence of pharmacologically important phytochemicals.

Table 1 shows the body weight, absolute and relative testes weights of the experimental groups. F group showed significantly decreased relative testes weight by 13.7% compared to C group. However administration of AEMO resulted in 50% recovery from the altered organ weight. The relative testes weight of C+MO did not deviate from that of C.

The extent of LPO and the levels of GSH, GR and GST are shown in table 2. The activities of SDH, SOD and CAT are shown in table 3. F group showed 34.3% increase in LPO and 31.7% decrease in GSH levels compared to C group. The activities of antioxidant enzymes GR and GST were decreased by 34.5 and 26.7% respectively and SOD and CAT were decreased by 43.9 and 52.3% respectively in F group compared to C. In contrast, the activity of SDH, the polyol pathway enzyme, was increased by 31.8% in F group compared to C. The C+MO group showed significantly decreased LPO by 13.8% when compared to C group. Administration of AEMO for 60 days totally prevented the tissue LPO in F+MO group and the GSH levels were restored to normal with 100% recovery. F+MO group showed 68.2 and 100% recovery in GR and GST activities and 100 and 91% recovery in SOD and CAT activities respectively (Fig-1). The control group which received the AEMO also showed a significant increase in the activities of GR, SOD and CAT activities.

The photomicrograph of section of testes of experimental rats are shown in Fig. 2(a—d). The control rat testis shows normal histological picture. The seminiferous tubules (ST) are uniformly arranged with well defined interstitial tissue (IT). The spermatogenesis pattern appears to be normal with fully matured sperms in the centre of ST. C+MO group also shows normal picture of testis. However moderate fatty changes are visible in F group which shows enlarged ST with increased lumen size, heavy fat deposition and mild degeneration. The spermatogenesis pattern appears abnormal and a reduction in the number of fully matured spermatozoa (hypospermatosis) is seen in F group when compared to C group. However F+MO group

showed improved histological picture. The size of the ST was decreased and the spermatogenesis pattern appears to be rectified partially. Though the ST showed fat deposition the intensity seems to be less when compared to F group.

Fig 1: Percent recovery from fructose fed alterations in LPO, GSH levels and antioxidant enzyme activities in testes of experimental rats.

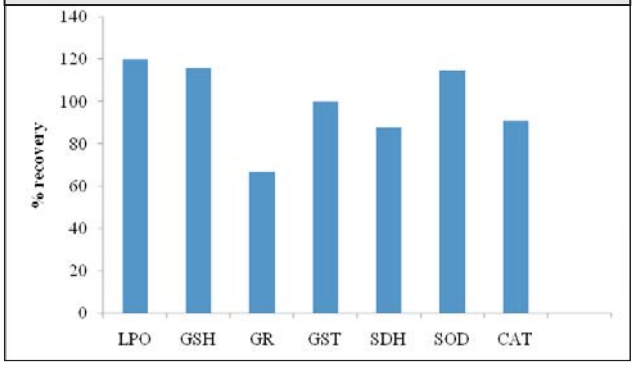

Fig 2a Photomicrograph of section of Testis of C group: Seminiferous tubules (ST) are uniformly arranged with well defined interstitial tissue (IT), orderly arranged germ cells and plenty of sperms at the centre of ST.

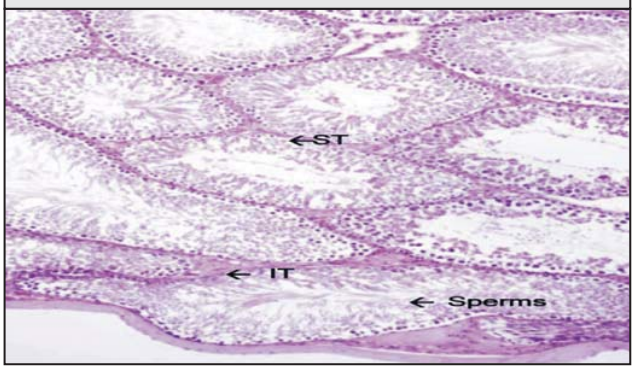

Fig 2b. Photomicrograph of section of testis of C+MO group. Seminiferous tubules (ST) are uniformly arranged with well defined interstitial tissue (IT), orderly arranged germ cells and plenty of sperms at the centre of ST.

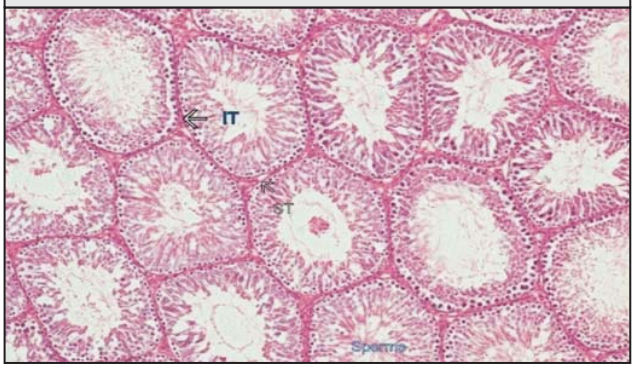

Fig 2c. Photomicrograph of section of of testis F group. Seminiferous tubules (ST) are enlarged with increased lumen size. Heavy fat deposition is seen in ST and the spermatogenesis pattern appears abnormal with a reduction in number of sperms.

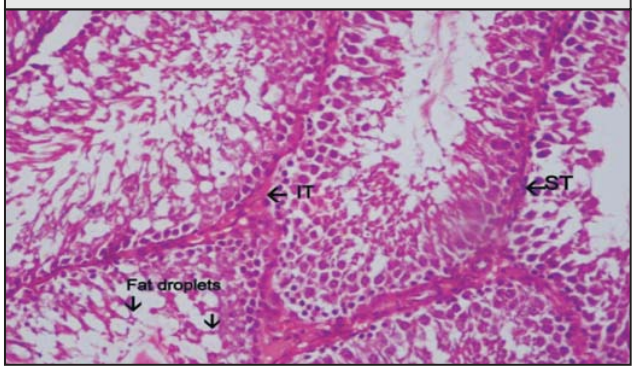

Fig 2d . Photomicrograph of section of of testis F+MO group. Seminiferous tubules (ST) show reduced lumen size and reduced fat deposition compared to F group. Spermatogenesis pattern appears partially rectified.

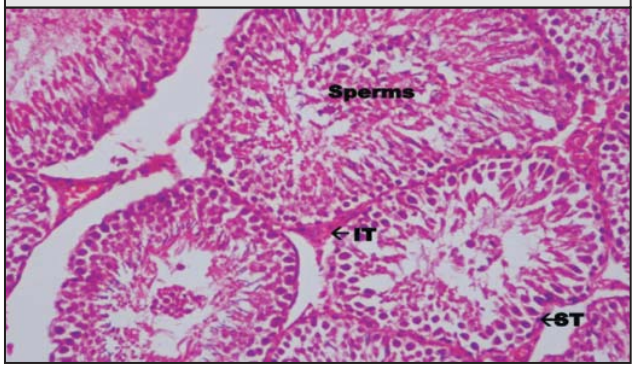

Table 1: Effect of AEMO on the relative organ weight of testes in experimental rats.

Group→	C	C+MO	F	F+MO
Body weight(g)	278±2.29[a]	277±2.29[a]	305±2.00[b]	280±1.42[a]
Testes weight(g) (Absolute)	3.46±0.07[a]	3.45±0.06[a]	3.22±0.04[b]	3.31±0.05[c]
Relative weight	1.24±0.02[a]	1.25±0.02[a]	1.07±0.02[b]	1.18±0.02[c]

Values are mean ± S.E.M., (n= 6 animals). Values with different superscripts with in the row are significantly different at $p<0.05$ (Duncan's Multiple Range Test).

Table 2: Effect of AEMO administration on LPO, GSH, GR and GST in testes of experimental rats.

Group→ Parameter ↓	C	C+MO	F	F+MO
LPO(A)	10.3±0.10[a]	8.8± 0.08[b]	13.8± 0.07[c]	9.6± 0.10[d]
GSH(B)	3.6±0.02[a]	4.3±0.01[b]	2.4±0.01[c]	3.8±0.02[d]
GR (C)	6.0±0.02[a]	6.4±0.01[b]	3.9±0.04[c]	5.3±0.01[d]
GST(D)	215.8±0.54[a]	214.3±0.64[a]	169.1±0.85[b]	215.2±0.58[a]

A: n mol MDA formed/min/mg protein; B: µg/mg protein; C: µmol NADPH oxidised/min/mg protein; D: µmol CDNB-GSH conjugate formed/min/mg protein.

Values are mean ± S.E.M., (n= 6 animals). Values with different superscripts with in the row are significantly different at p<0.05 (Duncan's Multiple Range Test).

Table 3: Effect of AEMO administration on the activities of SDH, SOD and CAT in testes of experimental rats.

Group→ Parameter ↓	C	C+MO	F	F+MO
SDH(A)	4.9±0.02[a]	5.0±0.04[b]	6.4±0.04[c]	5.1±0.02[b]
SOD(B)	22.2±0.25[a]	23.9±0.21[b]	12.4±0.15[c]	23.6±0.22[b]
CAT(C)	1.7±0.02[a]	1.8±0.02[b]	0.82±0.01[c]	1.64±0.01[d]

A: µmol NADH oxidised/min/mg protein, B: units/mg protein, C: µmol of H_2O_2 consumed/min/mg protein.

Values are mean ± S.E.M., (n= 6 animals). Values with different superscripts with in the row are significantly different at p<0.05 (Duncan's Multiple Range Test).

DISCUSSION

High fructose diet has prooxidant effects [33]. High fructose fed rats have shown increased free radical production by mechanisms like autoxidation of glucose, enhanced glycation and altered polyol pathway [34]. Metabolic stress resulting from changes in energy metabolism also plays a role in enhanced oxidative stress. Increased or enhanced catabolism of fructose would result in energy depletion in cells making them more susceptible to peroxidation [35].

Further the susceptibility of tissues to oxidative stress may depend on alterations in lipid composition. Enhanced lipid accumulation, observed in the testes of fructose fed rats, has contributed to the increased LPO in these animals. Several studies have also shown increased concentrations of ROS and oxidative stress in fructose fed rats [36]. Rabbani et al [37] reported increased oxidative stress in testes of type 2 diabetic model.

In the present study an increased LPO was observed in testes of F group but administration of AEMO totally prevented the increased tissue LPO in F+MO rats. The lipid lowering and insulin sensitizing effects of AEMO along with its antihyperglycemic affect[1] could have brought a favourable metabolic environment avoiding the prooxidant conditions with reduced oxidative stress in F+MO group. The C+MO group also showed decreased LPO compared to C group which clearly indicates that AEMO prevented the age related oxidative stress too. In vitro and *ex-vivo* studies have revealed that the water extract of *M. oleifera* leaf significantly inhibited TBARS formation in $CuSo_4$ induced rabbit and human LDL oxidation [38].

The lyophilized hydroalcoholic extract of *M. oleifera* leaf prevented the increase in lipid peroxidation in rats with Isoproterenol induced myocardial damage [39].

Depleted GSH content and decreased activities of GR and GST are explained by the enhanced activity of SDH, a polyol pathway enzyme. Polyol pathway leads to generation of NADH and depletion of $NADPH_2$. NADH is a substrate for NADH oxidase to generate ROS and $NADPH_2$ is required by GR to regenerate GSH. Depleted plasma GSH and tissue GSH was well documented in fructose diet induced insulin resistant rats [40]. Decreased activity of GST in F group could also be explained by the low content of GSH since GSH is a substrate and cofactor of GST [41]. Earlier studies also revealed decreased activities of GSH dependant enzymes in fructose fed insulin resistant rats [42]. However, AEMO treatment activated the compensatory mechanism against the oxidative stress which was reflected by enhanced activiities of GR and GST in F+MO group

SOD and CAT are widely distributed in all animal cells. SOD a Cu/Zn containing enzyme is a major defense for aerobic cells in combating the toxic affect of superoxide radicals[43]. Catalase, a haemoprotein, reduces H_2O_2 produced by dismutation reaction and prevents generation of hydroxyl radical. F group showed

decreased activities of SOD and CAT when compared to C group. Earlier studies also indicated decreased activities of antioxidants both GSH dependant and GSH independent [44]. Restoration of SOD activity in F+MO reveals an efficient defense against superoxide radicals known to inactivate CAT.

Studies on *M. oleifera* leaf on their antioxidant and radical scavenging property are well documented [45], [46].

Much of the research in diabetes is concentrated on tissues related to metabolic derangements, insulin production and IR ie., pancreas, liver, skeletal muscle, kidney and heart. Less attention has been paid to studies related to the reproductive system in diabetes.

In the present study fructose fed rats show abnormal histological alterations of testes which are consistent with those of previous studies [47]. Clinical and experimental studies have shown that diabetes mellitus has adverse effects on male sexual and reproductive functions in humans and animals [48], [49]. Impairment of spermatogenesis, reduced sperm count, sperm motility, seminal fluid volume and low testosterone levels were found in diabetic subjects [50].

The testes are sensitive to environmental exposure induced cellular damage. Apoptosis, known as programmed cell death, is a form of cell death that serves to eliminate dying cells in proliferating or differentiating cell population. Apoptosis control is critical for normal spermatogenesis in the adult testes [51]. The oxidative stress is recognized as a strong mediator of apoptosis. Diabetic condition was reported to enhance apoptosis of germ cells [52] and also increased LPO was reported to impair membrane functions by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors [53]. Thus in the current study the increased oxidative stress with compromised antioxidant status has led to the tissue damage in F group. However, the administration of AEMO improved the

histological picture in F+MO group. Lilibeth [17] reported that the administration of hexane extract of *M. oleifera* enhanced male reproduction in mice. Some herbs like *Tribulus terresteris* also showed beneficial effects and improved testicular function in rats[54].Numerous epidemiological studies suggest that herbs/diets rich in phytochemicals and antioxidants execute a protective role in health and disease [55]. *Moringa* leaves act as a good source of natural antioxidant due to the presence of various types of antioxidant compounds such as ascorbic acid, flavonoids, phenolics and carotenoids. Moreover , trace elements which play an important role in radical scavenging and antioxidant system and the presence of phytochemicals may have protected the F+MO rats against the adverse effects of high fructose diet.

Thus our results clearly show that AEMO exhibited protective role with its enhanced radical scavenging activity and antioxidant potential which could exert a beneficial action against pathological alterations in testes caused by oxidative stress under IR conditions.

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