Original article:

Testicular oxidative stress in Sprague-Dawley rats treated with bitter melon (*Momordica charantia*): the effect of antioxidant supplementation

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Abstract

***Objective*:** An important mediator of testicular injury is oxidative stress; the implicating pathway has been pointed at a free radical mechanism by researchers. This article, investigates the effect of bitter melon *(Momordica charantia)* (MC) seed extract and antioxidant supplementation in the testes of Sprague-Dawley (S-D) rat. ***Methodology*:** Ninety male S-D rats, weighing between 110-214 g, were assigned randomly into six main Groups A to F. Group A was administered 50 mg/100 g of MC extract orally, between 6 to 16 weeks. Group B were pre-treated with ascorbic acid (AA) 0.01mg/kg, three days/week, α-tocopherol (AT) 20 mg/kg, five days/week and both test solutions (TS) i.e. AA and AT; 0.01 and 20 mg/kg, three and five days/week for 8 weeks. This was followed by administration of the extract at dose and duration as in A. Group C received the extract for 8 weeks and afterwards post-treated for another 8 weeks with AA, AT and both TS (as above). Group D in addition to the extract administration were treated with AA, AT and both TS in dose and duration similar to B above. Group E had AA, AT and both TS alone for 8 weeks. Group F served as the control subjects. The animals testicular tissues were processed for malondialdehyde (MDA) and AA concentrations. Serum testosterone (TT) assay was done from left ventricular blood. ***Results*:** The extract administered for 6, 8 and 16 weeks produced significantly ($p < 0.05$) increased testicular MDA (1.74 ± 1.15, 1.84 ± 0.38 and 2.38 ± 0.40) compared to control (0.38 ± 0.02, 0.38 ± 0.03 and 0.35 ± 0.02) and decreased AA (0.01 ± 0.02, 0.01 ± 0.01 and 0.00 ± 0.01) compared to control (0.15 ± 0.02, 0.12 ± 0.02 and 0.13 ± 0.02). There was also an associated significant decrease ($p < 0.05$) in peripheral TT levels compared to control. The extract produced responses that showed no prophylactic rather modulatory effect with TS. ***Conclusion*:** These findings suggest that the extract resulted in changes in the testicular oxidative status. This may play a role in testicular dysfunction that may compromise fertility.

Key words: *Momordica charantia,* malondialdehyde, ascorbic acid, testosterone.

Introduction

Over seven decades ago, α-tocopherol (AT) was recognized as a powerful lipophilic antioxidant that is absolutely vital for the maintenance of mammalian spermatogenesis [1]. Ascorbic acid (AA) contributes to the support of spermatogenesis at least in part through its capacity to reduce AT and maintain this antioxidant in an active state [2]. Free oxygen radicals are known to possess ability to react with cellular macromolecules such as nucleic acids, lipids, proteins and carbohydrates to produce a destructive effect [3]. For example oxygen radicals have a destructive effect on lipids (lipid peroxidation) of all membranes. The end product of this phenomenon is called malondialdehyde (MDA). It is a reliable and generally accepted indicator of lipid peroxidation [3]. The levels can increase to the extent that it cumulates into a situation known as oxidative stress [3, 4, 5]. Free oxygen radicals or reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, and the hydroxyl ion are molecules that contain an oxygen atom. A free radical is any chemical

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species capable of independent existence and contains one or more unpaired electrons. They are highly reactive due to the presence of unpaired valence shell electrons. The cellular structures of membranes are prevented from the damaging effect of ROS by systems that scavenge the free radicals from the cellular environment [4].

There is normally an intricate balance between these amount of free radicals generated and scavenged by a cell with damage occurring when the equilibrium is disturbed [6]. Thus when there is increased production of ROS, cellular structures are vulnerable to the effects of oxidative stress [4]. Spermatozoa are rich in polyunsaturated fatty acids and this makes them susceptible to attack by ROS or membrane lipid peroxide ions. The equilibrium between the amount of ROS produced and scavenged is related to the stability and damage of the gamete cell[4]. Free radicals are implicated to have detrimental effects on sperm functions, which depend on its nature and concentration [7]. Antioxidants are substances that inhibit the destructive effects of oxidation by the ROS in the body [8]. Numerous antioxidants include ascorbic acid, alpha tocopherol, beta carotene and melatonin. Other known antioxidants include enzymes such as superoxide dismutase catalase and gluthatione peroxidase are credited to ROS detoxification [9]. The antioxidant properties of *Momordica charantia* has been pointed out previously by researchers [10] but its pro-oxidant effect on the testes is yet to be described. Research has proven that at a high dose antioxidant could act as pro-oxidant releasing free radicals [11]. This present study was thus designed to determine the possible role of oxidative stress of *Momordica charantia* on the testes as a mode of contraception in male S-D rats previously described.

Materials and methods

Test solutions
The Test solutions (TS) used were the antioxidants α-tocopherol (AT) and ascorbic acid (AA) at doses 20 mg/kg [12] and 0.01

mg/kg [13] respectively.The doses were calculated based on the animal's individual weekly body weights and aliquots approximated to the nearest numeral administered. It was done using insulin syringe (100 IU equivalent to 1ml) via intramuscular (i.m.) route.

Collection and identification
The ripe fruits of MC harvested in June were purchased from the local market in Lagos. It was authenticated by Professor J. Olowokudejo, a taxonomist in the Department of Botany, University of Lagos, where the voucher specimen was deposited (ascension number FHI 108422).

Preparation of seed extract
The seeds were dried in an oven (temperature of between 30-40°C) for a week. The dried seeds were weighed and Soxhlet extraction done using alcohol and water as solvents at the Pharmacognosy department of College of Medicine, University of Lagos. The percentage yield was 23.0% w/w, from which a dose of 50 mg/100 g of body weight was calculated and administered orally.

Sources and maintenance of Rats
Ninety male S-D rats 6-8 weeks old were used in this study. The rats were procured from the Laboratory Animal Centre of the College of Medicine of the University of Lagos and were authenticated by a taxonomist [14] in the Department of Zoology of the University of Lagos. They were kept in plastic cages in the Animal Room of the Department of Anatomy and allowed to acclimatize for two weeks under standard laboratory conditions of temperature 27-30°C. Lighting was by natural daylight such that the rats were exposed to approximately 12:12 light–dark cycle. They were fed with commercially available rat chow (Livestock feeds Plc, Ikeja, Lagos, Nigeria) and had unrestricted access to water.

Experimental protocol
The animals were randomly allocated into 6 main groups A to F. Which were further sub-divided into 3 sub-groups (A_1 to A_3; B_1 to B_3; C_1 to C_3; D_1 to D_3; E_1 to E_3 and F_1 to F_3) of 5 rats. Subgroups A_1 to A_3 indicate different

treatment durations of 6, 8 and 16 weeks administered a single oral dose of 50 mg/100 g of MC extract. Group B pre-treated with TS (B_1: 0.01 mg/kg of AA, three times a week Mondays, Wednesdays and Fridays; B_2: 20 mg/kg of AT, five days a week Mondays, Tuesdays, Wednesdays, Thursdays and Fridays while B_3: both TS) for 8 weeks and then fed the extract at dose and duration as in A. Subgroups C_1 to C_3 received the extract for 8 weeks (as in Group A) and afterwards post-treated for another 8 weeks with AA, AT and both TS (as in Group B). Group D comprise rats receiving the extract and TS simultaneously for a duration of 8 weeks. Therefore in addition to the extract D_1 to D_3 were treated with AA, AT and both TS in dose and duration similar to B above. Subgroups E_1 to E_3 had AA, AT and both TS. Finally, Group F animals were used to compare events in the other groups was administered distilled water throughout the experiment.

At the end of the different experimental durations, the animals were sacrificed. The testes were assessed for malondialdehyde and ascorbic acid concentration, *cauda epididymal* fluids were processed for sperm count and motility. Testicular morphometry (weight and volume) was also assessed. The protocol was approved by ethical committee of the institute.

Testicular malondialdehyde concentration

Testicular tissue samples weighing 0.25 g were homogenized in 2.5 ml of 0.15 M potassium chloride. The supernatant from the homogenate was collected and stored at 20^0C. The MDA levels were determined as described by Buege and Aust (1978) [15]. A 2ml aliquot of 0.375% Trichloroacetic acid- Thiobarbituric acid-Hydrochloric acid (TCA-TBA-HCL) was added to 1.0ml of the supernatant of testicular tissue homogenate. This was mixed vigorously and heated for 15minutes in a water bath at 80-90^0C. The sample was cooled in ice cold water again for 15 minutes at 1500 *g* and the tubes were placed in the photometer and absorbance taken at 535nm against the reagent blank. Concenration was calculated using the molar absorptivity of malondialdehyde which is 1.56×10^5 $M^{-1}cm^{-1}$.

Testicular ascorbic acid concentration

Testicular AA concentrations were determined as by the Association of Official Analytical Chemist (1990) [16]. This method is based on the oxidation of ascorbic acid to dehydro ascorbic acid, which when heated with dinitrophenylhydrazine forms a coloured complex with absorption maxima at 520 nm. Briefly, 0.5 g testicular tissue sample was homogenized in 12.5 ml of 0.5% oxalic acid for 10 minutes. The homogenate was centrifuged at 1000 g and the supernatant collected. 1.5 ml of 4% trichloroacetate and 1.0 ml of 2, 4-dinitrophenylhydrazine were added to 0.5 ml of supernatant in test tubes. The tubes were then incubated at 50^oC for 1 hour. With the tubes in ice-bath, 1.25 ml of 85% sulphuric acid was added drop-wise with mixing after each drop. The tubes were removed from the ice-bath and left at room temperature for 30 minutes. The absorbance was then read at 520 nm after setting the spectrophotometer to zero with the blank. The concentration of ascorbic acid was calculated using the formula: Concentration of ascorbic acid = $Abs_{(test)}$ X $Con_{(std)}/Abs_{(std)}$ Where, $Abs_{(test)}$ is the absorbance for the sample, $Con_{(std)}$, concentration of standard ascorbic acid and $Abs_{(std)}$, absorbance of standard ascorbic acid (0.086), derived from Beer-Lambert law (Absorbance proportion to Concentration) (Association of Official Analytical Chemists, 1990).

Testosterone assay

Serum TT was assayed from blood obtained from a left ventricular puncture. The samples were spun at 3000 *g* for 10 min in an angle head centrifuge at 25 ^{o}C. The samples were assayed in batches from a standardized curve using the Enzyme Linked Immunosorbent Assay (ELISA) method [17]. The microwell kits used were from Syntro Bioresearch Inc., California USA. Using 10 µl of the standard, the samples and control were dispensed into coated wells. 100 µl TT conjugate reagent was added and then 50 µl of anti-TT reagent. The contents of the microwell were thoroughly mixed and then incubated for 90 minutes at room temperature. The mixture was washed in distilled water and further incubated for 20 minutes. The reaction was stopped with 100 µl

of 1N hydrochloric acid. Absorbance was measured with an automatic spectrophotometer at 450 nm. A standard curve was obtained by plotting the concentration of the standard versus the absorbance and TT concentration was determined from the standard curve.

Table I: Malondialdehyde and Ascorbic acid concentration in experimental and control Sprague-Dawley rats

Groups ($n = 90$)		Testicular MDA (nmol/g of testis x 10^{-7})	Testicular AA (mg/100 m^3 of testis)
A_1	Wk 6	1.74 ± 1.15^b	0.01 ± 0.02^b
A_2	Wk 8	1.84 ± 0.38^b	0.01 ± 0.01^b
A_3	Wk 16	2.38 ± 0.40^b	0.00 ± 0.01^b
B_1	$AA_{8wks} - MC_{8wks}$	2.12 ± 0.08^b	0.02 ± 0.02^b
B_2	$AT_{8wks} - MC_{8wks}$	2.35 ± 0.80^b	0.04 ± 0.06^b
B_3	$AA_{8wks}, AT_{8wks} - MC_{8wks}$	1.73 ± 0.34^b	0.02 ± 0.02^b
C_1	$MC_{8wks} - AA_{8wks}$	0.31 ± 0.08	0.10 ± 0.02
C_2	$MC_{8wks} - AT_{8wks}$	0.69 ± 0.45	0.08 ± 0.04
C_3	$MC_{8wks} - AA_{8wks}, AT_{8wks}$	0.45 ± 0.20	0.12 ± 0.02
D_1	$MC_{8wks} + AA_{8wks}$	0.37 ± 0.05	0.19 ± 0.02
D_2	$MC_{8wks} + AT_{8wks}$	0.59 ± 0.22	0.16 ± 0.02
D_3	$MC_{8wks} + AA_{8wks}, AT_{8wks}$	0.30 ± 0.009	0.21 ± 0.09
E_1	AA_{8wks}	0.34 ± 0.08	0.14 ± 0.05
E_2	AT_{8wks}	0.39 ± 0.18	0.12 ± 0.05
E_3	AA_{8wks}, AT_{8wks}	0.36 ± 0.12	0.11 ± 0.06
F_1	Distilled water$_{6wks}$	0.38 ± 0.02	0.15 ± 0.02
F_2	Distilled water$_{8wks}$	0.32 ± 0.03	0.12 ± 0.02
F_3	Distilled water$_{16wks}$	0.35 ± 0.02	0.13 ± 0.02

Values expressed as Mean ± standard deviation; $^b p < 0.05$; Distilled water given for 6 to 8 weeks; MDA: Malondialdehyde; MC_{8wks}: 50 mg/100 g of Momordica charantia extract fed for 8wks; AA_{8wks}: 0.01 of ascorbic acid treated for 8wk; AT_{8wks}: 20 mg/kg of α-tocopherol treated for 8wk; AA_{8wks}, AT_{8wks}: α-tocopherol & ascorbic acid at doses 0.01 and 20 mg/kg administered simultaneously for 8wk; wk: weeks

Statistical analysis

Results were expressed as mean±SD. Analysis was carried out using analysis of variance (ANOVA) with Scheffe's post hoc test. The level of significance was considered at $p < 0.05$.

Results

Testicular malondialdehyde levels

There was a marked duration dependent statistically significantly ($p < 0.05$) increase in testicular MDA concentration compared to control (Table-I). The mean values for animals fed distilled water and MC extract for 6, 8 and 16 weeks were 0.38 ± 0.02, 0.32 ± 0.03, 0.35 ± 0.02 *vs*1.74 ± 1.15, 1.84 ± 0.38, 2.38 ± 0.40 respectively. This increase is also same for those pre-treated with AA, AT, TS for 8 weeks and later post-treated with the extract for another 8 weeks, mean values were 2.12 ± 0.08, 2.35 ± 0.80, 1.73 ± 0.34. Animals fed the extract for 8 weeks followed by treatment with AA, AT and both TS for 8 week were 0.31 ± 0.08, 0.69 ± 0.45, 0.45 ± 0.20 respectively, when compared to control (0.38 ± 0.02, 0.32 ± 0.03, 0.35 ± 0.02) showed no significant difference ($p < 0.05$; Table-I). The mean MDA for animals post treated with AT was observed to be slightly higher values were not statistically significant. Also showing no significant difference from control (0.38 ± 0.02) were the groups in which the extract was administered concurrently with AA, AT, TS for 8 weeks viz 0.37 ± 0.05, 0.59 ± 0.22, 0.30 ± 0.01 (Table-I). Finally MDA values for rats treated with AA, AT, and TS for 8 weeks alone were similar to control.

Testicular ascorbic acid concentration levels
The mean AA values followed an inverse relationship to MDA concentration. It showed a significant ($p < 0.05$) duration dependent decrease in testicular AA form a control of 0.15 ± 0.02, 0.12 ± 0.02, 0.13 ± 0.02 to 0.01 ± 0.02, 0.01 ± 0.01 and 0.00 ± 0.01 for animals fed the extract for 6, 8 and 16 weeks. Also those pre-treated with AA, AT, TS for 8 weeks and later post-treated with the extract for another 8 weeks (0.02 ± 0.02, 0.04 ± 0.06, 0.02 ± 0.02) compared to control. The testicular AA showed substantial recovery to base line control value in rats fed the extract for 8 weeks thereafter treated with AA, AT and both TS for 8 weeks (0.10 ± 0.02, 0.08 ± 0.04, 0.12 ± 0.02; $p < 0.05$; Table-I).

The group in which the extract was administered concurrently with AA, AT and TS for 8 weeks, showed an appreciable modulation (Table-I). These showed no significant difference. Lastly, AA values for rats treated with AA, AT, and TS for 8 weeks alone were similar to control.

Table II: Serum Testosterone levels in experimental and control Sprague-Dawley rats

Groups ($n = 75$)		Testosterone (ng/ml)
B_1	$AA_{8wks} - MC_{8wks}$	0.13 ± 0.02^b
B_2	$AT_{8wks} - MC_{8wks}$	0.12 ± 0.07^b
B_3	$AA_{8wks}, AT_{8wks} - MC_{8wks}$	0.17 ± 0.09^b
C_1	$MC_{8wks} - AA_{8wks}$	0.32 ± 0.35
C_2	$MC_{8wks} - AT_{8wks}$	0.35 ± 0.24
C_3	$MC_{8wks} - AA_{8wks}, AT_{8wks}$	0.34 ± 0.21
D_1	$MC_{8wks} + AA_{8wks}$	0.31 ± 0.08
D_2	$MC_{8wks} + AT_{8wks}$	0.34 ± 0.20
D_3	$MC_{8wks} + AA_{8wks}, AT_{8wks}$	0.37 ± 0.24
E_1	AA_{8wks}	0.38 ± 0.06
E_2	AT_{8wks}	0.33 ± 0.15
E_3	AA_{8wks}, AT_{8wks}	0.37 ± 010
F_1	Distilled water$_{6wks}$	0.36 ± 0.02
F_2	Distilled water$_{8wks}$	0.31 ± 0.09
F_3	Distilled water$_{16wks}$	0.29 ± 0.01

Values expressed as Mean $\pm$ standard deviation; $^b p < 0.05$; Distilled water given for 6 to 8 weeks; MC_{8wks}: 50 mg/100 g of Momordica charantia seed extract fed for 8wks; AA_{8wks}: 0.01 of ascorbic acid treated for 8wk; AT_{8wks}: 20 mg/kg of α-tocopherol treated for 8wk; AA_{8wks}, AT_{8wks}: α-tocopherol & ascorbic acid at doses 0.01 and 20 mg/kg administered simultaneously for 8wk; wk: weeks.

Testosterone concentration
The serum TT level following administration of the extract for 6, 8 and 16 weeks were observed to diminish markedly. It decreased from 0.36 ± 0.02 (control) to 0.15 ± 0.09 (after 8 weeks) and 0.05 ± 0.02 ng/ml (after 16 weeks; Figure 1). These values only become significant ($p < 0.05$) after the 8 weeks. A similar reduction in serum TT levels was also observed in rats pre-treated prophylactically with AA, AT and both TS for 8 weeks followed by the extract for another 8 weeks (Table-II). There was no significant ($p < 0.05$) difference observed in serum TT in rats administered the extract and antioxidant concurrent and with TS treatment alone compared to control (Table-II).

Discussion

It has been shown previously from research that administration of AA to normal animals stimulates both sperm production and TT secretion [18]. It is also known that AA counteracts the testicular oxidative stress induced by exposure to pro-oxidant substances such as arsenic, cadmium, endosulfan and alcohol [19, 20]. In this present study, animals fed

the extract alone (Group A) and those pre-treated with TS before administering the extract (Group B) had both showed a high level of testicular lipid peroxidation. This means the extract triggered oxidative stress via the release of free radicals in the testes, as evidenced by the elevation of testicular MDA and also a decrease in testicular AA in these groups. The treatment with AT has also been shown to suppress lipid peroxidation in testes [21] and reverse detrimental effects of oxidative stress on testicular function [22, 23]. This finding is in concert with our results, where the extract resulted in a decreased in testicular AA level. There is a high possibility that the extract may have acted via production of oxidative stress, in view of the fact that co-administration of AA, AT or both TS with MC (Group D) were found to protect against the elevation of MDA levels as values were identical to control. It was also observed that both TS and AA offered a better protection against the oxidative stress from MC than AT when concurrently administered with the extract compared to their control counterpart. The reason for these differential actions of these two vitamins cannot be fully explained. However, it is possible for AA to be acting in somewhat unexplained mechanism(s) in addition to its action as an antioxidant. It is known that AA is necessary for steroidogenesis [24] and has a co-enzymatic function in the biosynthesis of steroid hormones, such as testosterone [21]. Our finding of a decreased serum TT level in Group A rats fed the extract may not be unconnected with the effect of MC. Studies have shown that the serum TT level correlates positively with sperm concentration and motility [25, 26]. It is also known that sperm production cannot proceed to an optimal completion without a continuous TT supply [27]. Our result demonstrated that MC produced a significant reduction in serum TT level and therefore, could be linked to a cessation of spermatogenesis. A resulting decreased spermatogenesis and increased sperm damage secondary to the oxidative stress induced by MC at the level of testicular micro-environment could therefore be correctly extrapolated. Also the MDA resulting from the membrane damage can also induced further sperm damage have shown in previous studies [28]. Thus animals in these groups were expected to show a diminished fertility.

In Conclusion, the present study showed that MC exerted its effect via generation of free radicals with accompanying decrease in serum TT, when administered at an oral dose of 50mg/100 g body weight of rat. This effect was also observed to be dose dependent.
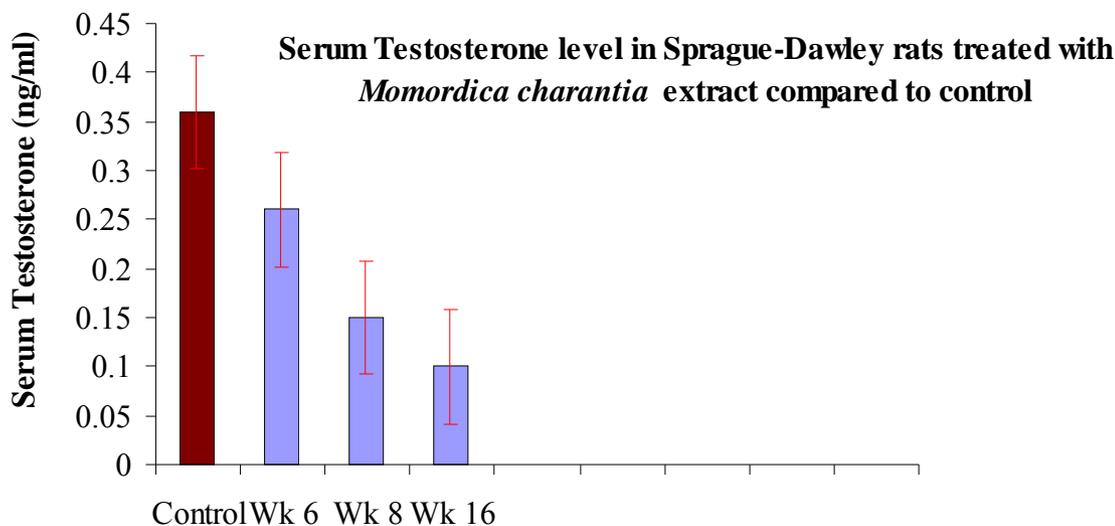

Figure 1: Serum testosterone level in Sprague-Dawley rats treated with *Momordica charantia* extract compared to control

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