Nitrite-induced testicular toxicity in rats: therapeutic potential of walnut oil

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ABSTRACT

Objective: To determine the impact of walnut oil on nitrite-induced testicular toxicity in Sprague-Dawley (SD) rats. Available evidence suggests that walnut oil contains high levels of important unsaturated fatty acids including alpha-linolenic acid (ALA) and omega-3; nitrite is a reproductive toxicant that causes the loss of germ cells in the seminiferous tubules and generates oxidative stress in the testes, thus reducing sperm counts and affecting sperm morphology.

Methods: This study included 24 male and 24 female adult SD rats. The male rats randomly assigned to Group A (controls) were given normal saline 2 ml/kg. The rats in Groups B, C, and D were given 50mg/kg body weight (bwt) of walnut oil, 0.08 mg/kg bwt of nitrite, and 0.08 mg/kg bwt of nitrite + 50 mg/kg of walnut oil respectively for 28 days via gastric gavage. Tested parameters included: testicular histology, sperm parameters, reproductive hormones, fertility, malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione, and catalase (CAT).

Results: A severe decrease in spermatogenic cell series, hypocellularity, tubular atrophy, decreased sperm quality, and increased MDA levels were observed in the rats given nitrite only when compared to controls. Rats given 50 mg/kg of walnut oil had significant growth of seminiferous epithelium compared to controls. The rats given walnut oil and nitrite had significant growth of seminiferous epithelium, improved sperm quality, and had decreased MDA levels.

Conclusion: Walnut oil attenuated the deleterious effects of nitrite to the testes, reduced oxidative stress, and promoted spermatogenesis.

Keywords: spermatogenesis, nitrite, testis, Hormone, oxidative stress

INTRODUCTION

Walnut oil is a good source of omega-3 fatty acids that are essential for human nutrition (Iwamoto et al., 2002). The major fatty acids found in walnut oil are linoleic, oleic, and linolenic acid (Tsamouris et al., 2002). The preventive roles of monounsaturated fatty acids and poly cyclic unsaturated fatty acids (MUFA and PUFA) in cardiovascular diseases have been identified (Tavakoli et al., 2005). It has been reported that the consumption of walnut (kernel and oil) lowers blood cholesterol levels (Damascono et al., 2011; Rajaram et al., 2009). Studies have shown that walnut oil has antioxidant properties and reduces the risk of coronary heart disease and inflammation, in addition to being useful in the treatment of skin diseases and high blood pressure (Labuckas et al., 2008; Fladman, 2002; Reiter et al., 2005). Walnut is also effective in the treatment of type-2 diabetes and enhances cardiovascular flexibility (Tapsell et al., 2009). It has been reported that due to its high concentration of natural antioxidants, walnut can be consumed as a protection against certain types of cancer (Bostani et al., 2014). It may also reduce the risk of cardiovascular disease (Miraliakbari & Shahidi, 2008; Yang et al., 2009).

Nitrate is one of the most common contaminants in rural and suburban areas due to its high solubility in water; the contamination of ground water by nitrate originates primarily from fertilizers, septic systems, and manure storage or spreading operations. (McCasland et al., 1985). Nitrite is a normal component of human diet found in most vegetables (Dennis & Wilson, 2003). Spinach and lettuce may contain as much as 2500 mg/kg of the compound, followed by 302.0mg/kg in curly kale, 61.0mg/kg in green cauliflower, and 13mg/kg in asparagus. Nitrite levels in 34 vegetable samples including different varieties of cabbage, lettuce, spinach, parsley, and turnips ranged between 1.1 and 57mg/kg (Correia et al., 2010; Leszczyńska et al., 2009). Fresh meat contains 0.4-0.5mg/kg of nitrate and 4-7mg/kg of nitrate (Dennis & Wilson, 2003). The presence of nitrite in animal tissue is a consequence of the metabolism of nitric oxide (Meulemans & Delsenne, 1994). Nitrite can be reduced to nitric oxide or ammonia by many species of bacteria (Cymeryng et al., 1998). Under hypoxic conditions, nitrite may release nitric oxide, which causes potent vasodilation. Nitrate (NO-), and its chemical cousin Nitrite (NO2), can cause methemoglobinemia or blue baby syndrome (Kostić et al., 1998). High nitrate levels may also indicate the presence of other pollutants, such as bacteria or pesticides, as these may follow the same path as nitrate into the water supply (McCasland et al., 1985). It has been reported that inorganic nitrate and inorganic nitrite inhibited steroidogenesis in mouse Leydig tumor cells (MLTC-1) (Panesar, 1999; Panesar & Chan, 2000). Both nitrite and nitrate can endogenously be converted to nitric oxide (NO) (Ellis et al., 1998). The inhibitory effects of nitrite and nitrate occur through the action of the metabolite nitric oxide (NO), which is an inhibitor of steroid hormone synthesis (Masuda et al., 1997; Natarajan et al., 1997).

The present study focused on the salutary role of walnut oil in nitrite-induced testicular toxicity in Sprague-Dawley rats.

MATERIALS AND METHODS

Collection of plants and preparation of walnut oil

Walnuts were collected from Igbara oke, Ondo State, Nigeria and were identified and authenticated in the Department of Agronomy, Ladoke Akintola University of Technology, Ogbomoso, Nigeria; a voucher specimen of the plant was deposited for future reference. After cleaning and drying in the shade, the air-dried walnut kernels
were weighed using a CAMRY (EK50SS, Indian) electronic scale and milled in an automatic electrical blender (model FS-323, China) to powdered form. Then, a portion of powdered walnut was kept in solvent n-hexane in the laboratory; after 24 hours in the solvent, the sample was strained and the solution obtained was poured into a rotary device (Rotavapor® model ED-100) at 40-50°C to let the solvent evaporate. To ensure the removal of moisture, the samples were kept in a vacuum desiccator (GCD-064X, KIKO, Japan) for an additional 24 hours. At the end of the process, a bright yellow oily substance with a density of 1.1485 gr/ml was obtained.

Animal care and experimental design
A total of 24 male and 24 female adult Sprague-Dawley rats were used in this study. Twenty-four male Sprague-Dawley rats of the first filial generation were randomly assigned to three treatment groups identified as B, C and D or the control group A (n = 6 in each group). Orogastric tubes were used to administer the following to the animals in treatment groups B, C and D, respectively: 0.08 mg/kg body weight nitrite; 50mg/kg body weight walnut oil and 0.08mg/kg body weight of nitrite; and 50mg/kg body weight of walnut oil for 28 days. The animals in the control group (group A) were administered equal amounts of phosphate buffered saline (PBS). All the animals were housed in clean, well-ventilated cages measuring 34.0x20.5x20.0cm (temperature: 28-31°C; humidity: 50-55%) (Yakubu et al., 2008). The cages were cleaned daily. All animals were checked for illnesses, abnormal behavior, and morphological anomalies. All experimental procedures followed the recommendations provided in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health (NIH, 1985). The rats were fed with standard chow at a recommended daily dose of 100 g/kg as advised by the International Centre of Diarrheal Disease Research, Bangladesh (ICDDR, B). Drinking water was supplied ad libitum. The weights of the rats were documented at procurement, during the period of acclimatization, at commencement of administrations, and once a week throughout the period of the experiment, using a CAMRY electronic scale (EK50SS, Indian).

Surgical procedure
Twenty-four hours after the last administration, the rats were given intraperitoneal pentobarbital sodium (40mg/kg) and their peritoneal cavities were opened through a lower transverse abdominal incision. Then the testes of the rats in the control and experimental groups were immediately removed. The weights of the testes from each group were recorded. The animals were decapitated between 9:00 and 11:00 AM, and blood samples were collected. The blood samples were centrifuged at 4°C for 10 min at 250xg and the serum obtained was stored at -20°C until assayed. The harvested testis specimens were fixed in Bouin's fluid for histological analysis (Avwioro, 2010).

Epididymis sperm count, viability and motility
Spermatozoa from the cauda epididymis were released by cutting into 2ml of medium (Hams F10) containing 0.5% bovine serum albumin (Feng et al., 2001). After 5 min of incubation at 37°C (with 5% CO2), the cauda epididymis sperm reserves were determined using a hemocytometer. Sperm motility was analyzed with a microscope (Leica DM750) and reported as the mean number of motile sperm according to the method described by the WHO (WHO, 1999).

Biochemical estimations
Lipid peroxidation products were estimated by measuring TBARS and were determined in accordance with the method published by Niehaus & Samuelsson (1968). Non-enzymatic antioxidants such as reduced glutathione (GSH) and catalase (CAT) were estimated as described by Ellman (1959) and Sinha (1972), respectively. SOD activity in the testes was determined according to the method described by Marklund & Marklund (1974).

Hormone determination
The hormonal profiles of endocrine markers testosterone (TT), follicle stimulating hormone (FSH) and leuteinizing hormone (LH) were measured using commercially available immunoassay (ELISA) kits (Randox Laboratories Ltd, Admore Diamond Road, Crumlin, Co., Antrim, United Kingdom, Qt94QY) according to manufacturer instructions.

Testicular histology preparation
The testes were harvested and fixed in Bouin's fluid for 24h and were then transferred to 70% alcohol for dehydration. The tissues were passed through 90% and absolute alcohol and xylene for different durations before they were transferred into two changes of molten paraffin wax for 1 hour each in an oven at 65°C for infiltration. They were subsequently embedded and serial sections cut on a rotary microtome set at 5 microns. The tissues were picked up with albuminized slides and allowed to dry on hot plates for 2 min. The slides were dewaxed with xylene and passed through absolute alcohol (two changes), 70% alcohol, 50% alcohol, and then water for 5 min. The slides were then stained with Hematoxylin and Eosin. The slides were mounted in DPX. Photomicrographs were taken at a magnification of 100x on a Leica DM750 microscope.

Morphometric studies
Morphometric studies were carried out as reported by Akang et al. (2015). The primary aim was to estimate the volumes of seminiferous tubule epithelium (seminiferous epithelium) and interstitium in the testes. This was done in accordance with Howard & Reed (2005) and Baines et al. (2008). Four sections per testis and six microscope fields per section were randomly chosen for analysis. Fields were sampled as images captured on a Leica DM750 bright field microscope (Germany) via LAZ software. Volume densities of testicular ingredients were determined by randomly superimposing a transparent grid comprising 35 test points arranged in a quadratic array. Test points falling on a given testis and its ingredients were summed over all fields from all sections. The total number of points hitting on a given ingredient (lumen (EL), epithelium (EE), interstitium (EI)), divided by the total number of points hitting on the testis sections (ET) multiplied by 100, provided an unbiased estimate of its percent volume density/volume fraction.

Fertility Testing
Fertility testing was performed by a modification of the method reported by Ligha et al. (2012). Each male rat was isolated, placed in a cage and paired with a pro-estrous female rat in the first hours of the estrous cycle as determined by vaginal smear examination. On the following day, the female rats were checked after mating to detect spermatozoa in their vagina by microscopic examination of the vaginal fluid. Females in which sperm plugs were detected the following morning after mating were assumed to be on day one of gestation. The fetuses were removed by ventral laparotomy on the 21st day of gestation and counted.
Data presentation and statistical analysis
Data were expressed as Mean±SEM. Statistical differences between the groups were evaluated by one-way ANOVA, followed by Dunnett’s comparison test to compare between treated and control groups. Differences yielding \( p<0.05 \) were considered statistically significant. Statistical analyses of data were performed using GraphPad Prism 5 Windows (GraphPad Software, San Diego, California, USA).

RESULTS

Testes and body weight

The results are listed in Table 1. There was a significant decrease in the mean testis weight in the nitrite treated group compared with controls (\( p<0.05 \)). There were no significant differences in testis weight between the other groups. The bodyweight of the rats given 50 mg/kg body weight of walnut increased (242.3±6.75) when compared to controls (237.3±7.25), while the body weight of the rats given nitrite (181.7±7.91) and nitrite + walnut oil (212.0±6.25) decreased significantly (\( P<0.05 \)). There were no significant changes in the proportions of sperm abnormalities in the experimental groups compared with controls (Table 1).

Sperm motility, viability and count
Nitrite treatment significantly decreased sperm count, motility, and viability in the nitrite group compared with controls and other experimental groups. Sperm count, motility, and vitality were 34.39±2.85, 28.19±3.28 and 51.86±3.36, respectively, in the nitrite treated group. The corresponding values in the walnut oil group were 72.69±3.17, 73.41±4.75, and 82.63±3.12, and the corresponding values in the nitrite + walnut oil group were 66.54±3.53, 65.69±4.06, and 68.11±2.26. However, sperm count, motility, and vitality in controls were 75.58±3.56, 70.04±4.95, 80.33±2.86. There were no significant changes in the proportions of sperm abnormalities in the experimental groups compared with controls (Table 1).

Antioxidant (SOD, GSH, CAT) and MDA levels
As shown in Table 2, MDA levels in the nitrite group increased when compared with the control group and decreased in the nitrite + walnut oil group in relation to the nitrite group. Antioxidant levels (SOD, GSH, and CAT) decreased significantly in the nitrite group (*\( p<0.05 \)) when compared to controls; SOD, GSH and CAT levels in the nitrite + walnut oil group decreased (*\( p<0.05 \)) when compared to controls.

| Table 1. Effect of walnut oil on testis weight, body weight, and sperm parameters of nitrite treated rats |
|---------------------------------------------------------------|
| **Parameters**                                                | **Groups**                                                                 |
|                                                             | A (control) | B (50 mg/kg bwt walnut oil) | C (0.08 mg/kg bwt Nitrite) | D (0.08 Nitrite + 50 walnut oil) mg/kg bwt |
| Testis weight (g)                                            | 1.52±0.05   | 1.45±0.07                   | 1.03±0.04*                  | 1.39±0.06                                  |
| Body weight (g)                                              | 237.3±7.25  | 242.3±6.75\(^*\)            | 181.7±7.91*\(^*,\)         | 212.0±6.25\(^*\)                          |
| Sperm count (x10⁶/ml)                                        | 75.58±3.56  | 72.69±3.17\(^*\)            | 34.39±2.85\(^*,\)          | 66.54±3.53\(^*\)                          |
| Motility (%)                                                 | 70.04±4.95  | 73.41±4.75\(^*\)            | 28.19±3.28\(^*,\)          | 65.69±4.06\(^*\)                          |
| Viability (%)                                                | 80.33±2.86  | 82.63±3.12\(^*\)            | 51.86±3.36\(^*,\)          | 68.11±2.26\(^*\)                          |

Values are expressed as Mean ± S.E.M, \( n=6 \) in each group
\(^*\): significantly different from the control group
\( β \): significantly different from the nitrite group
\( α \): significantly different from the walnut oil group
\( p<0.05 \), One-Way ANOVA.

bwt: body weight

| Table 2. Effects of walnut oil on antioxidant levels and lipid peroxidation of nitrite treated rats |
|---------------------------------------------------------------|
| **Parameters**                                                | **Groups**                                                                 |
|                                                             | A (control) | B (50 mg/kg bwt walnut oil) | C (0.08 mg/kg bwt Nitrite) | D (0.08 Nitrite + 50 walnut oil) mg/ kg bwt |
| Malondialdehyde (MDA) (nmol/mg)                              | 5.17±0.46   | 3.49±0.20\(^*,\)            | 8.15±0.55\(^*\)            | 3.05±0.36\(^*,\)                          |
| Super oxide dismutase (SOD), (u/mg protein)                  | 12.08±0.75  | 13.03±0.81\(^*\)            | 4.39±0.33\(^*,\)           | 11.16±0.57\(^*\)                          |
| Glutathione peroxidase, (umol/mg protein)                    | 9.64±1.04   | 8.75±0.97\(^*\)             | 4.17±0.21\(^*,\)           | 7.91±0.89\(^*\)                          |
| Catalase, (u/mg protein)                                     | 19.93±1.23  | 21.45±1.38\(^*\)            | 11.19±0.87\(^*,\)          | 17.51±1.24\(^*\)                          |

Values are expressed as Mean ± S.E.M, \( n=6 \) in each group
\(^*\): significantly different from the control group
\( β \): significantly different from the nitrite group
\( α \): significantly different from the walnut oil group
\( p<0.05 \), One-Way ANOVA.

bwt: body weight
**Hormonal assay**

In comparison with controls (2.87±0.09), the rats in the nitrite group had significantly lower TT (1.06±0.05) (p<0.05) levels, while the LH and FSH (1.16±0.08 and 0.73±0.16) levels were not significantly changed (1.40±0.07 and 0.86±0.19). In the walnut oil and nitrite + walnut oil group, TT (2.91±0.08 and 2.63±0.10) as well as FSH (1.33±0.06 and 0.91±0.20) and LH (1.22±0.07 and 0.86±0.17) levels were not significantly different from the values seen in controls for TT (2.87±0.09), FSH (1.40±.07) and LH (0.86±0.19) (Fig. 1).

**Morphometric analysis**

After the 28 days of the study, the volume density of germinal epithelium of controls (64.90±0.89) was significantly different from that of the rats given nitrite (58.08±0.33) (p<0.05). However, there was a significant increase in the walnut oil (69.18±0.22) and nitrite + walnut oil (70.66±0.22) groups when compared to controls (64.90±0.89) and rats given nitrite alone (58.08±0.33). Lumen density significantly decreased in the nitrite group (9.60±0.23) compared to the control group (15.42±0.20). The interstitium had a significant increase in the nitrite group (9.60±0.23) (p<0.05). One-Way ANOVA. n=6 in each group.

**Testicular histology**

Sections of the seminiferous tubules of control rats had moderately circular or oval outlines with normal stratified seminiferous epithelium showing cells of the spermatogenic series and spermatooza within the lumen (Fig. 2A). The rats in the walnut oil group showed normal cellular composition in their germinal epithelium with sperm cells in the lumen and a normal interstitium and prominent Leydig cells (Fig. 2B). The seminiferous tubules of the rats treated with nitrite alone showed severe reduction of cells of the spermatogenic series, hypcellularity in the interstitium, widening of the tubular lumen, tubular atrophy, and fewer spermatooza in the tubular lumen (Fig. 2C). The rats in the nitrite and walnut oil groups showed cells of the spermatogenic series and normal cellular composition in their germinal epithelium with sperm cells in the lumen and a normal interstitium (Fig. 2D).

**Fertility testing in control and treated rats**

The rats treated with 0.08mg/kg body weight of nitrite suffered with decreased fertility potential, as more than 90% of the female rats they mated with did not get pregnant. By their turn, the group given 0.08mg/kg body weight of nitrite and 50mg/kg body weight of walnut oil did not suffer such negative effects, since all the female rats they mated with got pregnant and had at least six fetuses. There was a decrease in the number of fetuses produced in group B treated with 50mg/kg body weight of walnut oil and 0.08mg/kg body weight of nitrite + 50mg/kg body weight of walnut oil when compared to controls. The number of pregnancies and fetuses was significantly decreased in group C rats given nitrite when compared with controls and rats in groups B and D (p<0.05) (Table 4).

**DISCUSSION**

Herbal remedies are alternative medications prepared from plants and plant extracts used to treat illnesses and diseases and to address psychological concerns. Herbal remedies have been around for centuries and are precursors to modern medicine (Burke et al., 2006). Herbal remedies are obtained from a wide variety of natural sources including plant leaves, bark, berries, flowers, and roots (Khaki et al., 2011). Walnut oil is reported to be a good source of omega-3 fatty acids that are essential for human nutrition (Ozcan et al., 2010). In this study there was a great concern that exposure to nitrite might cause reproductive toxicity in rats. Our results showed that nitrite administration decreased the relative weights of the testes and the body weight of the included rats as reported in previous studies (Akcintunde et al., 2014). At the end of our study, nitrite administration had significantly reduced sperm count, motility, and viability by subjecting the spermatooza to increased oxidative stress-induced damage, since their plasma membranes contain large quantities of polyunsaturated fatty acids (PUFAs) (Bansal & Bilaspuri, 2010; Alvarez & Storey, 1995) and their cytoplasm contains low concentrations of scavenging enzymes (Saleh & Agarwal, 2002; Sharma & Agarwal, 1996). Increased formation of ROS has been correlated with decreased sperm motility (Atkken et al., 1989; Armstrong et al., 1999).

The link between ROS and reduced motility may be due to a cascade of events that result in rapid loss of intracellular ATP leading to axonemal damage and sperm immobilization (Bansal & Bilaspuri, 2010; de Lamirande & Gagnon, 1995). Our investigation also demonstrated that exposure to nitrite decreased testosterone concentrations, indicating interference with steroidogenesis. Administration of walnut oil increased testosterone levels and indicated the positive effect of walnut oil on the hypothalamic-pituitary-testicular axis. The hypothalamic-pituitary-testicular axis can be affected by various negative and positive feedback mechanisms. Nitric oxide (NO) is one of the factors affecting this axis. High levels of arginine in walnut can be converted to nitric oxide. Nitric oxide increases the release of GnRH, which in turn increases gonadotropin secretion by activating the production of neuronal nitric oxide synthase in the pituitary gland (Barnes et al., 2002; Pinilla et al., 2001). Nitric oxide activates guanylate cyclase that causes the release of cyclic guanosine monophosphate, which by
Table 3. Morphometric analysis of testes after 28 days of treatment

| Parameters        | Groups                                      |
|-------------------|---------------------------------------------|
|                   | A (control)                                |
|                   | B (50 mg/kg bwt walnut oil)                |
|                   | C (0.08 mg/kg bwt Nitrite)                 |
|                   | D (0.08 Nitrite + 50 walnut oil) mg/kg bwt |
| Germinal epithelium (%) | 64.90±0.89                                 |
|                   | 69.18±0.37*                                |
|                   | 58.08±0.33*  α                            |
|                   | 70.66±0.20*  β                            |
| Lumen (%)         | 15.42±0.20                                 |
|                   | 12.52±0.18*                                |
|                   | 9.60±0.23*  α  β                          |
|                   | 11.99±0.12*                                |
| Interstitium (%)  | 21.06±0.26                                 |
|                   | 16.91±0.27*  β                            |
|                   | 26.73±0.21*  α  β                          |
|                   | 20.39±0.71*                                |

Values are expressed as Mean ± S.E.M  
n=6 in each group  
*: significantly different from the control group  
β: significantly different from the nitrite group  
α: significantly different from the walnut oil group  
p<0.05. One-Way ANOVA.  
bwt: body weight

Figure 2. Section of the testis of control rat showing the seminiferous tubules containing cells of the spermatogenic series (SS) and the lumen (L) containing spermatozoa; Black arrow represents spermatogonium; P represents primary spermatocytes; Blue arrow represents spermatids and spermatozoa. (A-B) Section of the testis of rat treated with nitrite showing hypocellularity, reduction in cells of the spermatogenic series (SS) as a result of degeneration, sloughing and shortening of seminiferous epithelium; The seminiferous tubules show a single layer of basal spermatogonia; widened empty lumen (L); widened interstitium (I) due to tubular atrophy as a result of degeneration, Leydig cells show hyperplasia (brown arrows) and V shows vascular hemorrhage. (C) Section of the testis of rat treated with nitrite and walnut oil showing cells of the spermatogenic series and normal cellular composition in their germinal epithelium with sperm cells in the lumen and a normal interstitium. (H&E; X100).
eventually raising GnRH, LH and FSH, enhances sperm motility and induces erection in males (Miraoglia et al., 2011). Co-treatment with walnut oil prevented damage to the testes from nitrite exposure. This indicates nitrite interferes with walnut oil-related metabolic functions. The competitive mechanism of interaction is a plausible mechanism of protection offered by walnut oil against nitrite toxicity. This effect relates to the induction of oxidative stress. Our results showed that GPx, CAT, and SOD activities were distinctly lower in the testes of nitrite-exposed rats. Therefore, the increase in malondialdehyde (MDA), a by-product of lipid peroxidation (Dosumu et al., 2012; Saleh & Agarwal, 2002), observed in the present study might be due to the concomitant increase in the generation of free radicals such as H2O2 and OH in the testes of the nitrite-treated rats. This depicts an increase in lipid peroxidation. The interaction between nitrite and essential trace elements might be one of the reasons for decreased levels of antioxidant enzymes in rat testes.

In this study, walnut oil increased testicular antioxidant enzymes and decreased MDA levels when administered alone. Walnut oil also prevented the ravaging effects of nitrite on sperm parameters and testicular antioxidant enzymes when administered with nitrite. It has been reported that walnut contains significant amounts of antioxidants, omega-3 fatty acids and vitamin E, minerals, iron, sodium, calcium, magnesium, manganese, copper, potassium, phosphorus, protein, and fiber, which make it a varied nutritious meal (Cosmulescu et al., 2009). Therefore, it is reasonable to infer that the antioxidant constituents of walnut boosted the testicular non-enzymatic and enzymatic antioxidants to effectively scavenge free radicals and thus prevent lipid peroxidation. The consequence is hereby reflected in increased sperm count and motility. This finding is in accordance with the reports of Ojobor et al. (2017). Moreover, vitamin E, a chain-breaking non-enzymatic antioxidant also found in walnut, inhibits lipid peroxidation in membranes by scavenging peroxyl (RO•) and alkoxyl (ROO•) radicals (Akang et al., 2015; Saleh & Agarwal, 2002). The ability of vitamin E to maintain a steady state rate of peroxyl radical reduction in the plasma membrane depends on the recycling of vitamin E by external reducing agents such as ascorbate, found in walnut. The improved sperm parameters are also attributed to the abundant amount of vitamin E and zinc present in walnut oil, which are known male fertility agents as reported by Ajayieoba & Fadare (2006). Seeds of T. conophorum have been reported to contain reasonable amounts of zinc and vitamin E (Ojobor et al., 2015). Furthermore, our study showed histological abnormalities in the testicular tissue of rats given nitrite such as sloughing and shortening of the seminiferous epithelium, which led to decreased counts of cells of the spermatogenic series. This is in agreement with a previous study by Akintunde et al. (2014), in which nitrite led to the sloughing off of germ cells in the seminiferous tubules and evident increases in histological lesions in the seminiferous tubules and epithelial lining of the testes among tested rats (Akintunde et al., 2010). Interstitial hyperplasia and absence of Sertoli cells in the seminiferous lumen concur with the study of Pant & Srivastava (2000), who reported effects on the histopathology of the testes of adult male mice after exposure to 900ppm potassium nitrate via drinking water in a study evaluating the endocrine disrupting effects of in-utero exposure to nitrate in rats. It has been clear for decades that testosterone produced in the interstitial cells of Leydig is a necessary prerequisite for the maintenance of established spermatogenesis (Zirkin, 1998). The reduced cellularity of the interstitium in the testes of the rats treated with nitrite alone might have produced a decrease in testosterone and consequently poor spermatogenesis.

Walnut oil also maintained the histological architecture of the testes, increased the proliferative activity of spermatogonia, and maintained cells of the spermatogenic series when compared to controls. Walnut has been reported to contain reasonable amounts of zinc and vitamin E (Ayoola et al., 2011; Ojobor et al., 2015), which decrease lipid peroxidation. From our findings, when walnut oil was co-administered with nitrite, it protected the testes from the harmful effects of nitrite. This protective nature of walnut is enhanced by some of its phytochemical constituents, namely zinc and vitamin E, known for protecting cell membranes and for their scavenging effects on free radicals. In clinical trials, vitamin E supplementation has been found to increase fertilization rates possibly by improving membrane integrity and decreasing oxidative damage and lipid peroxidization potential (Comhaire et al., 2000; Geva et al., 1996). We therefore inferred from our observations that the antioxidants and micronutrients in walnut mitigated against the ravaging effects of nitrite on the testes.

The results observed in the rats given nitrite plus walnut oil suggested that the administration of walnut oil at the dosages and times of treatment used in our study decreased the interference nitrite would otherwise have had in the development and maturation of the male gonad, as illustrated by the fact that all females mated with them got pregnant. The improvement of fertility in the group treated with nitrite plus walnut oil showed that walnut oil acts as a powerful antioxidant to protect the oxidative stress of nitrite on the testes. Walnut has been reported to contain zinc and vitamin E, and the latter has been described as an excellent lipid soluble chain-breaking antioxidant (Traber & Atkinson, 2007).

**CONCLUSION**

In conclusion, we found that walnut oil effectively lowered nitrite-induced oxidative stress by reducing MDA.
levels and ameliorated the deleterious effects of nitrite on serum testosterone levels; however, it had no effect on serum FSH and LH levels, depleted the germinal epithelium, and caused hypocellularity and widening of the interstitium. Walnut oil did not only promote germinal epithelial growth, but also protected the cytoarchitecture of the testes from the damaging effects of nitrite. Walnut oil thus augmented spermatogenesis and defeated nitrite-induced oxidative stress through an antioxidant system of activities.

**CONFLICT OF INTEREST**

The authors have no conflict of interest to declare.

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**REFERENCES**

Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biol Reprod. 1989;41:183-97. PMID: 2553141 DOI:10.1095/biolreprod41.1.183

Ajajebo EO, Fadare DA. Antimicrobial potential of extracts and fractions of the African walnut - Tetracarpidium conophorum. Afr J Biotechnol. 2006;5:2322-5.

Aking EN, Oremosu AA, Osinubi AA, Dosumu OO, Kusemiju TO, Adelakun SA, Umaru ML. Histomorphometric studies of the effects of Telfairia occidentalis on alcohol-induced gonado-toxicity in male rats. Toxicol Rep. 2015;2:968-75. PMID: 28962436 DOI: 10.1016/j.toxrep.2015.06.009

Akintunde OW, Igbigbi PS, Olaniyi LWB. Adverse Effects on Male Rat Testes and Sperm Parameters of Orally Administered Nitrite. Intl J Dev Med Sci. 2010;3:27-35.

Akintunde OW, Adenowo TK, Kehinde BD. Some adverse effects of nitrite on oxidative status and histological structures of adult male wistar rats testes. Am J Res Commun. 2014;2:227-41.

Alvarez JG, Storey BT. Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. Mol Reprod Devel. 1995;42:334-46. PMID: 8579848 DOI: 10.1002/mrd.1080420311

Armstrong JS, Rajasekaran M, Chamulitrat W, Gatti P, Hellstrom WJ, Sikka SC. Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. Free Rad Biol Med. 1999;26:869-80. PMID: 10232830 DOI: 10.1016/S0891-5849(98)00275-5

Awwioro OG. Histology and Tissue Pathology. Principles and Techniques. 2nd ed. Ibadan: Claverianum Press; 2010.

Ayoola PB, Onawumi OO, Faboya OOP. Chemical evaluation and nutritive values of Tetracarpidium conophorum (Nigerian walnut) seeds. J Pharm Biomed Sci. 2011;11:1-5.

Baines H, Nwagwu MO, Hastie GR, Wiles RA, Mayhew TM, Ebling FJ. Effects of estradiol and FSH on maturation of the testis in the hypogonadal (hpg) mouse. Reprod Biol Endocrinol. 2008;6:4. PMID: 18230131 DOI: 10.1186/1477-7827-6-4

Bansal AK, Bilaspuri GS. Impacts of oxidative stress and antioxidants on semen functions. Vet Med Int. 2010;2010. pii: 686137. PMID: 20871827 DOI: 10.4061/2010/686137

Barnes MJ, Lapanowski K, Rafols JA, Lawson DM, Dunbar JC. Chronic nitric oxide deficiency is associated with altered leutinizing hormone and follicle-stimulating hormone release in ovariectomized rats. Exp Biol Med (Maywood). 2002;227:817-22. PMID: 12324663 DOI: 10.1177/15353702022700915

Bostani M, Aqababa H, Hosseini SE, Ashtiyani SC. A study on the effect of walnut oil on plasma levels of testosterone pre and post puberty in male rats. Am J Ethnomed. 2014;4:266-75.

Burke A, Smyth A, Fitz Gerald GA. Analgesic antipyretic agents. In: Goodman LS, Gilman A, Brunton LL, eds. The pharmacological basis of therapeutics. 11th ed. New York: McGraw-Hill; 2006. p. 637-731.

Comhaire FH, Christophe AB, Zalata AA, Dhooge WS, Mahmoud AM, Depuydt CE. The effects of combined conventional treatment, oralantioxidants and essential fatty acids on sperm biology in subfertile men. Prostaglandins Leukot Essent Fatty Acids. 2000;63:159-65. PMID: 10991774 DOI: 10.1016/plef.2000.0174

Correia M, Barroso A, Barroso MF, Soares D, Oliveira MBPP, Delerue-Matos C. Contribution of different vegetable types to exogenous nitrate and nitrite exposure. Food Chem. 2010;120:960-6. DOI: 10.1016/j.foodchem.2009.11.030

Cosmulescu SN, Baciu A, Achim G, Botu M, Trandafir I. Mineral Composition of Fruits in Different Walnut (Juglansregia L.) Cultivars. Not Bot Hort Agrobot Cluj. 2002;227:817-22. PMID: 12324663 DOI: 10.1016/j.foodchem.2009.11.030

Cymeryng CB, Dada LA, Podestá EJ. Effects of nitric oxide on rat adrenal zona fasciculata steroidogenesis. J Endocrinol. 1998;158:197-203. PMID: 9771463 DOI: 10.1677/joe.0.1580197

Damasceno NR, Pérez-Heras A, Serra M, Coñán M, Sala-Vila A, Salas-Salvadó J, Ros E. Crossover study of diets enriched with virgin olive oil, walnuts or almonds. Effects on lipids and other cardiovascular risk markers. Nutr Metab Cardiovasc Dis. 2011;21:S14-20. PMID: 21421296 DOI: 10.1016/j.numecd.2010.12.006

de Lamirande E, Gagnon C. Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. Hum Reprod. 1995;10:15-21. PMID: 8592032 DOI: 10.1093/humrep/10.suppl_1.15

Dennis MJ, Wilson LA. Nitrates and Nitrites. In: Caballero B, Finglas PM, Toldra F, eds. Encyclopedia of Food Sciences and Nutrition. San Diego: Academic Press; 2003. p. 4136-41.

JBRA Assist. Reprod. | v.23 | nº1 | Jan-Feb-Mar / 2019
Dosumu OO, Akinola OB, Akang EN. Alcohol-induced testicular oxidative stress and cholesterol homeostasis in rats - The therapeutic potential of virgin coconut oil. Middle East Fertil Soc J. 2012;17:122-8. DOI: 10.1016/j.mefs.2011.12.005

Ellis G, Adatia I, Yazdanpanah M, Makela SK. Nitrite and nitrate analysis: a clinical Biochemistry perspective. Clin Biochem. 1998;31:195-220. PMID: 964943 DOI: 10.1016/S0009-9120(98)00015-0

Ellman GL. Tissue sulphhydryl groups. Arch Biochem Biophys. 1959;82:70-7. DOI: 10.1016/0003-9861(59)90090-6

Feng R, He W, Ochi H. A new murine oxidative stress model associated with senescence. Mech Ageing Dev. 2001;122:547-59. PMID: 11295171 DOI: 10.1016/S0047-6374(01)00232-9

Fladman EB. The scientific evidence for a beneficial health relationship between walnuts and coronary heart disease. J Nutr. 2002;132:1062S-101S. PMID: 11983840 DOI: 10.1093/jn/132.5.1062S

Geva E, Bartoo B, Zabludovsky N, Lessing JB, Lerner-Geva L, Amit A. The effect of antioxidant treatment on human spermatozoa and fertilization rate in an in vitro fertilization program. Fertil Steril. 1996;66:430-4. PMID: 8751743 DOI: 10.1016/S0005-7774(00)00340-5

Howard CV, Reed MG, eds. Unbiased Stereology: Three-Dimensional Measurement in Microscopy. 2nd ed. Abingdon: Garland Science/BIOS Scientific; 2005.

Iwamoto M, Imaizumi K, Sato M, Hirooka Y, Sakai K, Takeshita A, Kono M. Serum lipid profiles in Japanese women and men during consumption of walnuts. Eur J Clin Nutr. 2002;56:629-37. PMID: 12080402 DOI: 10.1038/sj.ejcn.1601400

Khaki A, Fathiazad F, Nouri M, Khaki AA, Ghanbari Z, Ghanbari L, Amit A. The effect of antioxidant treatment on human spermatozoa and fertilization rate in an in vitro fertilization program. Fertil Steril. 1996;66:430-4. PMID: 8751743 DOI: 10.1016/S0005-7774(00)00340-5

Kostić T, Andrić S, Kovacević R, Marić D. The involvement of nitric oxide in the regulation of aldosterone synthesis by adrenal glomerulosa cells. J Steroid Biochem Mol Biol. 1997;61:47-53. PMID: 9328209 DOI: 10.1016/S0960-0760(97)00004-6

Kostić T, Andrić S, Kovacević R, Marić D. The involvement of nitric oxide in the regulation of aldosterone synthesis by adrenal glomerulosa cells. J Steroid Biochem Mol Biol. 1997;61:47-53. PMID: 9328209 DOI: 10.1016/S0960-0760(97)00004-6

Labuckas DO, Maestri DM, Perelli L, Martinez ML, Lamarque AL. Phenolics from walnut (Juglans regia L.) kernels: Antioxidant activity and interactions with proteins. Food Chem. 2008;111:421-7. PMID: 18781190 DOI: 10.1016/j.foodchem.2008.04.008

Niehaus WG Jr, Samuelsson B. Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem. 1968;6:126-73. PMID: 469943 DOI: 10.1016/S0009-9120(98)00007-0

NIH - National Institutes of Health. Guide for the Care and Use of Laboratory Animals: revised. Office of Science and Health Reports, DRR/NIH. Bethesda: DHEW Publication NIH - National Institutes of Health. Guide for the Care and Use of Laboratory Animals; 1985.

Ojobor CC, Chioma AA, Collins AC. Studies on the phytochemical and nutritional properties of Tetracarpidium conophorum (Black walnut) seeds. J Glob Biosc. 2015;4:1366-72.

Ojobor CC, Anosike CA, Ezeanyika LUS. Effect of Black Walnut (Tetracarpidium conophorum) Leaf Extract on the Reproductive Organ of Male Albino Rats. Int J Homeopath Nat Med. 2017;3:9-14. DOI: 10.11648/j.jijd.20170203.11

Özcan MM, Iman C, Arslan D. Physochemical properties, fatty acid and mineral content of some walnuts (Juglans regia L.) types. Agric Sci. 2010;2:62-7. DOI: 10.4236/as.2010.12009

Panesar NS. Role of chloride and inhibitory action of inorganic nitrate on gonadotropin-stimulated steroidogenesis in mouse Leydig tumor cells. Metabolism. 1999;48:693-700. PMID: 10381142 DOI: 10.1006/taap.2000.9079

Marklund S, Marklund G. Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem. 1974;47:469-74. PMID: 4215654 DOI: 10.1111/j.1432-1033.1974.tb03714

Masuda M, Kubota T, Karnada S, Aso T. Nitric oxide inhibits steroidogenesis in cultured porcine granulosa cells. Mol Hum Reprod. 1997;3:285-92. PMID: 9237255 DOI: 10.1093/molehr/3.4.285

McCasland M, Trautmann NM, Porter KS, Wagenet RJ. Nitrate: Health Effects in Drinking Water. Fact Sheet. Pesticide Safety Education Program. Cornell Cooperative Extension. 1985. Available at: http://psep.cce.cornell.edu/facts-slides-self/facts/nit-heef-grw85.aspx. Accessed: 11/07/2018.

Meulemans A, Delsenne F. Measurement of nitrite and nitrate levels in biological samples by capillary electrophoresis. J Chromatogr B Biomed Appl. 1994;660:401-4. PMID: 7866533 DOI: 10.1016/0378-4347(94)00310-6

Miraeglia E, De Angelis F, Gazzano E, Hassanpour H, Bertagna A, Aldieri E, Revelli A, Ghigo D. Nitric oxide stimulates human sperm motility via activation of the cyclic GMP/protein kinase G signaling pathway. Reproduction. 2011;141:47-54. PMID: 20965947 DOI: 10.1530/REP-10-0151

Mirallakbari H, Shahidi F. Antioxidant activity of minor components of tree nut oils. Food Chem. 2008;111:421-7. PMID: 20647445 DOI: 10.1016/j.foodchem.2008.04.008

Mirtle M, Trautmann NM, Porter KS, Wagenet RJ. Nitrate as a nitric oxide donor in cells of the bovine corpus luteum. Biol Reproduct. 1995;52:1191-4. PMID: 7570698 DOI: 10.1093/molehr/18.5.492

Natarajan R, Lanting L, Bai W, Bravo EL, Nadler J. The role of nitric oxide in the regulation of aldosterone synthesis by adrenal glomerulosa cells. J Steroid Biochem Mol Biol. 1997;61:47-53. PMID: 9328209 DOI: 10.1016/S0960-0760(97)00004-6

Orrego R, Cealetti G, Cealetti L, Verez D, Lechleider G. The effect of dietary nitrate and nitrite on superoxide anion radical formation in mouse placental cells. J Biol Chem. 1985;260:1366-72.

Ojobor CC, Chioma AA, Collins AC. Studies on the phytochemical and nutritional properties of Tetracarpidium conophorum (Black walnut) seeds. J Glob Biosc. 2015;4:1366-72.

Ojobor CC, Anosike CA, Ezeanyika LUS. Effect of Black Walnut (Tetracarpidium conophorum) Leaf Extract on the Reproductive Organ of Male Albino Rats. Int J Homeopath Nat Med. 2017;3:9-14. DOI: 10.11648/j.jijd.20170203.11

Özcan MM, Iman C, Arslan D. Physochemical properties, fatty acid and mineral content of some walnuts (Juglans regia L.) types. Agric Sci. 2010;2:62-7. DOI: 10.4236/as.2010.12009

Panesar NS. Role of chloride and inhibitory action of inorganic nitrate on gonadotropin-stimulated steroidogenesis in mouse Leydig tumor cells. Metabolism. 1999;48:693-700. PMID: 10381142 DOI: 10.1006/taap.2000.9079
Panesar NS, Chan KW. Decreased steroid hormone synthesis from inorganic nitrate and nitrate: studies in vitro and in vivo. Toxicol Appl Pharmacol. 2000;169:222-30. PMID: 11133344 DOI: 10.1006/taap.2000.9079

Pant N, Srivastava SP. Testicular and spermatotoxic effect of nitrate in mice. Hum Exp Toxicol. 2000;21:37-41. PMID: 12046722 DOI: 10.1191/096037102ht206oa

Pinilla L, González LC, Tena-Sempere M, Bellido C, Aguilar E. Effects of systemic blockade of nitric oxide synthases on pulsatile LH, prolactin, and GH secretion in adult male rats. Horm Res. 2001;55:229-35. PMID: 11740144 DOI: 10.1159/000050001

Rajaram S, Haddad EH, Mejia A, Sabaté J. Walnuts and fatty fish influence different serum lipid fractions in normal to mildly hyperlipidemic individuals: a randomized controlled study. Am J Clin Nutr. 2009;89:1657S-63S. PMID: 19339404 DOI: 10.3945/ajcn.2009.26736S

Reiter RJ, Manchester LC, Tan DX. Melatonin in walnuts: influence on levels of melatonin and total antioxidant capacity of blood. Nutrition. 2005;21:920-4. PMID: 15979282 DOI: 10.1016/nut.2005.02.005

Saleh RA, Agarwal A. Oxidative stress and male infertility: from research bench to clinical practice. J Androl. 2002;23:737-52. PMID: 12399514 DOI: 10.1002/j.1939-4640.2002.tb2324.x

Sharma RK, Agarwal A. Role of reactive oxygen species in male infertility. Urology. 1996;48:835-50. PMID: 8973665 DOI: 10.1016/S0090-4295(96)00313-5

Sinha AK. Colorimetric assay of catalase. Ann Biochem. 1972;47:389-94. PMID: 4556490 DOI: 10.1016/003-2697(72)90132-7

Tapsell LC, Batterham MJ, Teuss G, Tan SY, Dalton S, Quick CJ, Gillen LJ, Charlton KE. Long-term effects of increased dietary polyunsaturated fat from walnuts on metabolic parameters in type II diabetes. Eur J Clin Nutr. 2009;63:1008-15. PMID: 19352378 DOI: 10.1038/ejcn.2009.19

Tavakoli DA, Kimiagar SM, Velaei N. Persian Walnut effect on serum lipids in postmenopausal women. J Mazandaran Uni Med Sci. 2005;14:21-32.

Traber MG, Atkinson J. Vitamin E, antioxidant and nothing more. Free Radic Biol Med. 2007;43:4-15. PMID: 17561088 DOI: 10.1016/j.freeradbiomed.2007.03.024

Tsamouris G, Hatziantoniou S, Demetzos C. Lipid analysis of Greek walnut oil (Juglans regia L.). Z Naturforsch C. 2002;57:51-6. PMID: 11926543 DOI: 10.1515/znc-2002-1-209

WHO - World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. 4th ed. Cambridge: University Press; 1999.

Yakubu MT, Akanji MA, Oladiji AT, Adesokan AA. Androgenic potentials of aqueous extract of Massularia acuminata (G. Don) Bullock ex Hoyl. stem in male Wistar rats. J Ethnopharmacol. 2008;118:508-13. PMID: 18602232 DOI: 10.1016/j.jep.2008.05.020

Yang J, Liu RH, Halim L. Antioxidant and antiproliferative activities of common edible nut seeds. LWT-Food Sci Technol. 2009;42:1-8. DOI: 10.1016/j.lwt.2008.07.007

Zirkin BR. Spermatogenesis: its regulation by testosterone and FSH. Semin Cell Dev Biol. 1998;9:417-21. PMID: 9813188 DOI: 10.1006/scdb.1998.0253