Original Article

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DOI: 10.4103/jeca.jeca_11_17

Antioxidants: A therapy for cryptorchidism, true or false?

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Abstract:

BACKGROUND: Cryptorchidism remains a common congenital anomaly of the male genitalia, affecting 2.4%–5% of male infants.

AIM: This study investigates the effects of L-carnitine (LC), Biotin, and Vitamin D3 (Vit D) on the cryptorchid testis in Sprague-Dawley (SD) rats.

MATERIALS AND METHODS: Twenty-five adult male SD rats were divided into 5 groups of 5 each. The right testis of all rats was made cryptorchid. Group A received distilled water, Group B received LC, Group C received biotin, and Group D received Vit D while Group E received a cocktail of LC, Biotin, and Vit D. At the end 8 weeks, the animals were euthanized, and vital organs obtained, processed, and analyzed.

RESULTS: The testis of the untreated animals had marked depletion in the cells of the seminiferous tubules compared to those treated with antioxidants. They also had upregulated levels of malondialdehyde (MDA) which was inversely proportional to the levels of antioxidant enzymes. Epididymal sperm quality and testosterone level were also reduced in the untreated cryptorchid animals. These effects were, however, mitigated by the use of the antioxidants.

CONCLUSION: In this study, antioxidant therapy acted as a panacea for reversal of reactive oxygen species-induced male infertility in cryptorchid testis in SD rats. Further studies on antioxidants in comparison with hormonal supplements and surgical treatments of cryptorchid testis will be of great significance.

Keywords:

Antioxidants, biotin, cryptorchidism, free radicals, L-carnitine, spermatozoa, testis, Vitamin D

Introduction

Cryptorchidism is a very common congenital anomaly of the male genitalia, affecting 2.4%–5% of male infants and is more common in premature infants (Hutson *et al.*, 2010; Toppari *et al.*, 2010; and Fawzy *et al.*, 2015). Despite treatment by orchidopexy, the long-term effect remains problematic with the male fertility constantly threatened (Hadziselimovic and Herzog, 2001). Impaired fertility (33% in unilateral cases and 66% in bilateral undescended testes) and a cancer risk 5–10 times greater than normal are observed over time (Hutson *et al.*, 2010; Thorup *et al.*, 2010). About 10% of male infertility cases are linked with cryptorchidism and orchidopexy (Fawzy *et al.*, 2015). Likewise, the incidence of azoospermia is 13% in unilateral cryptorchidism (Chung and Brock, 2011).

The exact cause of cryptorchidism is elusive; however, it has been linked with androgen insensitivity as a result of environmental toxins (Cobellis *et al.*, 2014). Pregnant mothers exposed to compounds such as diethylstilboestrol are at high risk of cryptorchidism in the male child (Brucker-Davis *et al.*, 2003). In addition, the lower temperature of the scrotum against normal body temperature makes it favorable for optimal testicular

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How to cite this article: Oremosu AA, Osumah OG, Akang EN. Antioxidants: A therapy for cryptorchidism, true or false? J Exp Clin Anat 2017;16:87-92.

activities due to the thermoregulatory ability of the pampiniform plexus of veins and the testicular artery in the spermatic cord (Harrison and Weiner, 1949; Setchell, 1991). Hence, the high intra-abdominal temperature which is experienced by the cryptorchid testis adversely affects spermatogenesis and induces hyperproduction of reactive oxygen species (ROS), thereby increasing oxidative stress (Babaei *et al*., 2012, Ko *et al*., 2014). Furthermore, ROS hyperactivity over the testicular antioxidants will further lead to increased lipid peroxidation of the plasma membrane of the spermatozoa thereby, impairing capacitation and zeroing the spermatozoan ability to fertilize an oocyte (Akang *et al*., 2015; Guasti *et al*., 2017). Increased oxidative stress eventually leads to apoptosis (programmed cell death) of the spermatogenic cells, which explains the mechanism by which azoospermia is linked with cryptorchid testis (Kobayashi *et al*., 2013). Wang *et al*. (2007) observed a decline in sperm parameters, distorted testicular histology, and germ cell apoptosis after scrotal heating. They reported that apoptosis occurred mainly in the round spermatids and pachytene spermatocytes.

It has been suggested that the surgical correction of cryptorchidism (orchidopexy) between the ages of 6 months to 1 year may lower subfertility (Hadziselimovic, 2012). However, this early as possible intervention before 1 year of age is not a sure proof to the complete restoration of fertility. Howbeit, such individuals may still benefit from the assisted reproduction therapy. More worrisome is the treatment of cryptorchidism at puberty as such testis may be completely depleted of spermatozoa and may never function properly even after repair (Okuyama *et al*., 1989, Grasso *et al*., 1991).

Controversies exist about the use of endocrine agonist for the treatment of cryptorchid testis as against surgery or the combination of both (Fawzy *et al*., 2015). On the one hand, it is believed not to be necessary while on the other hand, it is believed to be adopted as the first line of treatment for cryptorchidism, failure of this treatment may warrant the second option-surgery (Mathers *et al*., 2009). It has been suggested that hormonal treatment is better to be considered when the biopsy of the undescended testis had no adult dark spermatogonic cells (Hadziselimovic and Hoecht, 2008, Thorup *et al*., 2012),

Antioxidant therapy has been demonstrated to be of great significance in the neutralization of ROS and in the management of oxidative stress-induced male infertility (Dosumu *et al*., 2012, Akang *et al*., 2015). A study by Azari *et al*. (2014) reported that Vitamin C was able to improve spermatogenesis in rat unilateral cryptorchid testis. More so, antioxidant therapy has been reported to increase testosterone levels in Sprague-Dawley (SD) rats (Akang *et al*., 2011; Dosumu *et al*., 2012). L-carnitine (LC) is a vitamin-like substance that is structurally similar to amino acids (Alzahrani, 2011). It is absorbed from foods through both active and passive transport across enterocyte (intestinal cell) membranes (Rebouche, 2004). It can also be synthesized endogenously from the essential amino acids lysine and methionine (Dokmeci *et al*., 2006). It is well known for its lipid lowering and antioxidant activities (Dokmeci *et al*., 2006, Salama *et al*., 2015). Biotin also known as vitamin H is also known to upregulate antioxidant enzyme levels and to effectively combat ROS (Al-Qudah and Ismail, 2012) Biotin is a water-soluble vitamin that serves as an essential coenzyme for carboxylases catalyzing the transfer of a carboxyl group to targeted substrates (Zempleni and Mock, 1999; Sedel *et al*., 2015). Vitamin D_3 (Vit D) which is currently being studied for its antioxidant property has been reported by Sharifi *et al*. (2014) as an adjunctive therapy to attenuate systemic inflammation and lipid peroxidation alongside other treatments. It has also been reported to attenuate DNA oxidative damage (Fedirko *et al*., 2010) Therefore, this study is aimed at determining the effects of LC, Biotin, and Vitamin D_3 on a unilateral cryptorchid testis in SD rats.

Materials and Methods

Experimental animals and protocols

Twenty-five male SD rats weighing between 200 and 220 g were randomly selected from the Nigerian Institute of Medical Research located in Yaba, Lagos. The animals were housed in the Laboratory Animal Center, College of Medicine, University of Lagos. The animal house was well ventilated with a temperature range of 28–32°C under day/night 12–12 h photoperiodicity. The rats were fed with standard rat chow (Pfizer Nig Ltd). They had access to water *ad libitum*. The experimental animals were further divided into five groups of 5 rats each. Group A: Control, received 1 ml of distilled water; Group B: 400 mg/kg body weight of LC (Zambrano *et al*., 2013); Group C: 5.0 mg/kg body weight of biotin (Sedel *et al*., 2015); Group D: 10 mg/kg body weight of Vit D (Fedirko *et al*., 2010); and Group E: A cocktail of the same doses of the 3 antioxidants (LC, Biotin, and Vit D). All animals in groups A-E were made unilaterally cryptorchid (right testis) for 8 weeks, and the entire administration was through the oral route. At the end of the experiment, the animals were euthanized, and vital organs were obtained for subsequent analysis. All experimental protocols followed the guidelines approved by the Ethics Committee of the College of Medicine, University of Lagos, Nigeria.

Induction of cryptorchidism

Cryptorchidism was induced in accordance with the method reported by Azari *et al*. (2014), the rats were anesthetized with intraperitoneal injection of 10 mg/kg body weight xylazine hydrochloride

(Alfasan Co., Netherlands) and 100 mg/kg body weight ketamine hydrochloride (Rotexmedica, Trittau, Germany). Then, the skin of the scrotal region was shaved and prepared with a povidone-iodine solution. The right inguinoscrotal region was incised, and the ligament of the tail of epididymis was separated. The freed testis was pushed back into the abdominal cavity through the internal inguinal ring. After which, the external inguinal ring was closed by 4/0 Nylon sutured to the muscle of the abdominal wall. The animals were left for 7 days postoperation to induce oxidative stress after which antioxidants were administered orally for 8 weeks.

Testicular histology preparation

The histology of the testes was done by the modification of method reported by Akang *et al*. (2015). The organs were harvested and fixed in Bouin's fluid for 24 h after which, it was 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 h each in an oven at 65◦C for infiltration. They were subsequently embedded and serial sections cut using rotary microtome at 5 microns. The tissues were picked up with albumenized slides and allowed to dry on a hot plate for 2 min. The slides were dewaxed with xylene and passed through absolute alcohol (2 changes); 70% alcohol, 50% alcohol and then to 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 x100.

Sperm count

This was done using the new improved Neubauer's counting chamber (Hematocytometer). The epididymal fluid was diluted with a normal saline solution by adding 0.9 ml–0.1 ml of the crushed epididymis. This chamber was then filled with sperm fluid and placed under a binocular light microscope using an adjustable light source. The ruled part was then focused and the number of spermatozoa counted in five 16-celled squares (Keel and Webster, 1990).

Sperm motility

The sperm motility analysis was carried out at room temperature using one epididymis of each rat. The percentage of sperm motility was calculated using the number of live sperm cells divided by the total number of sperm cells (motile and nonmotile), from two samples per epididymis of each rat. All sperm cells that were not moving at all were considered to be on motile while the rest, which displayed some movement, were considered to be motile (Yan *et al*., 2007; Oremosu and Akang, 2015).

Sperm morphology

Sperm morphology was evaluated by staining the sperm smears on microscope slides with two drops of 1% eosin stain after they were air dried. The slides were examined under the microscope under oil immersion with x100 objective. The abnormal sperm cells were counted, and the percentage calculated according to the method described by (Wyrobek and Bruce, 1978).

Hormone determination

The serum levels of rat-free testosterone were measured using commercially available enzyme-linked immunoassay kit (Elabscience) according to manufacturer's instructions.

Determination of testicular malondialdehyde and antioxidant enzymes levels

The MDA level was estimated by measuring TBARS and was determined by the method of Niehaus and Samuelsson (1968). Antioxidant enzymes such as reduced glutathione (GSH) and catalase were estimated by the methods of Ellman (1959) while superoxide dismutase (SOD) was measured by methods reported by Rukmini *et al*.(2004).

Statistics

The sperm parameters were log transformed to improve normality of data. Differences between groups were compared using one-way ANOVA and least significant difference *post hoc* test. This was done using the SPSS. The data were expressed as mean ± standard deviation.

Results

Histology of the testis

The cross section of the right cryptorchid testis of animals in Group A that received distilled water showed wide derangements in the germinal epithelium, distorted lumen with little or no spermatozoa. The other Groups B–E apart from Group C that received biotin and had a slight depletion of the spermatogenic cells, the rest had an intact germinal epithelium and interstitium with a marked presence of spermatozoa in the lumen [Figure 1].

Malondialdehyde and antioxidant enzymes

MDA levels significantly increased in the control animals (Group A) that received distilled water as compared to the other Groups (B–E) that received antioxidants [Table 1]. GSH and catalase levels significantly increased in animals that received Vit D (Group D) compared to Group A. SOD levels increased significantly in animals that received LC, Vit D and the cocktail of the 3 antioxidants (Groups B, D, and E) compared to the control [Table 1].

Sperm motility, sperm count, and sperm morphology analysis

The sperm motility increased in all groups; however, there was no significant difference when compared to Group A ($P > 0.05$). The sperm count of the animals that received biotin and those that received the cocktail

Table 1: Effect of antioxidants on testicular oxidative stress parameters

Groups (*n*=5)	tGSH (µmol/min)	tSOD (min/mg protein)	tCAT (µmol/mg protein)	tMDA (nmol/mL)
Group A	0.220±0.01	13.330±3.75	1.995±0.56	15.45±0.007
Group B	0.535±0.04	37.950±0.35*	5.825±1.79	7.30±0.001*
Group C	0.305±0.08	20.485±1.75	3.065±0.26	8.3±0.001*
Group D	0.770±0.34*	57.750±1.95*	8.635±1.80*	6.25±0.007*
Group E	0.665±0.04	49.970±1.06*	7.375±0.02	7.45±0.001*

Values are expressed as mean±SD. *P<0.05 compared with Group A. A - 1 mL of distilled water, B - 400 mg/kg of LC, C - 5 mg/kg of biotin, D - 10 mg/kg of Vit. D, E - Coctail of LC, biotin and Vit. D, tMDA - Testicular malondialdehyde, tSOD - Testicular superoxide dismutase, tGSH - Testicular Glutathione reductase, tCAT - Testicular catalase, SD - Standard deviation, LC - L-carnitine, Vit. D - Vitamin D_3

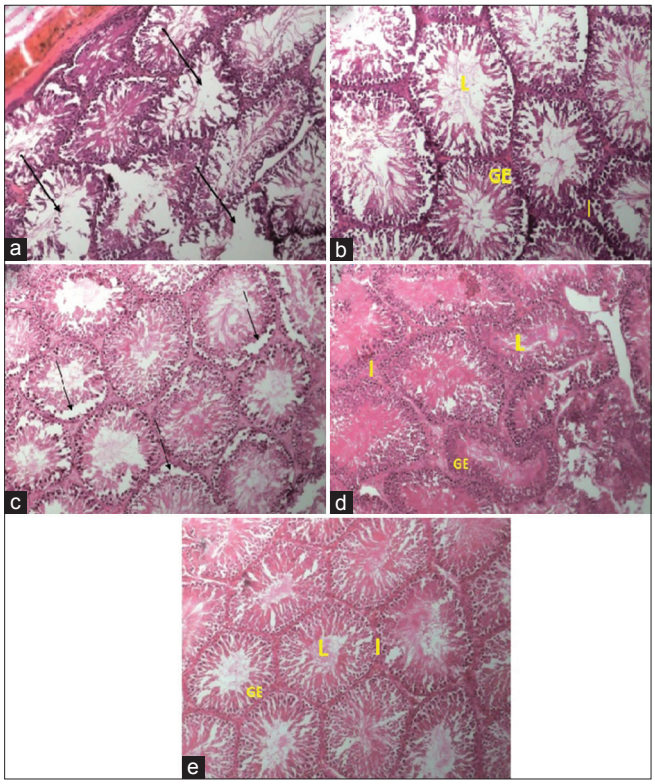

Figure 1: Testis with H and E stains ×100. (a) Control; received 1ml of distilled water. Arrows showing wide derangements in germinal epithelium. (b) received 400 mg/kg of L-carnitine; (c) received 5 mg/kg of Biotin; (d) received 10 mg/kg Vitamin D; (e) received a cocktail of L-carnitine, biotin, and Vitamin D; Groups B, D, and E depict a proper arrangement of spermatogenic cells within the germinal epithelium of each seminiferous tubule, with spermatozoa in the lumen (l) and Interstitial cells of Leydig (i) between each tubule. The arrows in group C depict slight derangements in germinal epithelium

of the 3 antioxidants significantly increased compared to control (P < 0.05). The sperm morphology of the abnormal spermatozoa was significantly lower in the group that received biotin compared to control [Table 2].

Testosterone levels

There was an increase in testosterone levels across the groups compared to control, but this increase was not significant (P > 0.05) as shown in Figure 2.

Discussion

Antioxidant therapy has been used to reduce heat-induced oxidative stress in the management of infertility

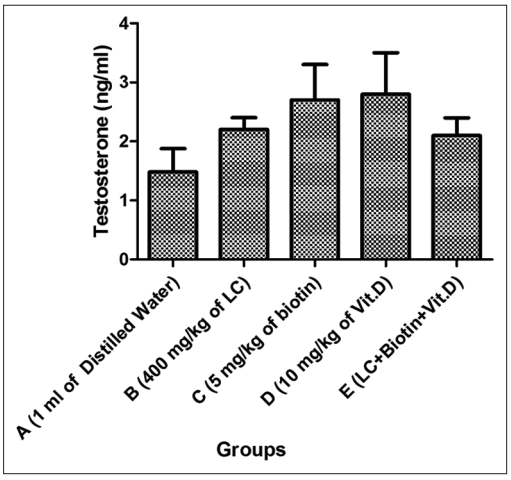

Figure 2: Effects of antioxidants on testosterone levels

(Ahmad *et al*., 2017). In this study, LC, biotin, and Vit D were efficacious in the reduction of lipid peroxidation induced by cryptorchidism. Lipid peroxidation which is exacerbated by the excess production of free radicals (ROS) over the total antioxidant levels (Ko *et al*., 2014) was increased with cryptorchidism. The animals that received antioxidant supplements had an increase in GSH, SOD, and catalase levels which was inversely proportional to MDA levels. These antioxidant enzymes neutralize the free radicals produced as a result of heat-induced testicular stress. Our result is in tandem with several reports supporting the use of antioxidants to mitigate oxidative stress-induced disorders (Ahmed *et al*., 2014, Salama *et al*., 2015, and Agarwal and Majzoub, 2017).

The negative effects of lipid peroxidation in the cryptorchid testis caused a marked reduction in sperm count and increased teratozoospermia and oligospermia. Oxidative stress is directly linked with upward regulation of apoptosis leading to the demise of spermatogenic cells (Koppers *et al*., 2011, Aitken *et al*., 2013, and Kobayashi *et al*., 2013). This may explain the derangements observed in the seminiferous epithelium of the cryptorchid testes in this study. More so, free radicals decrease the motility of the spermatozoon by increasing peroxidation of the plasma membrane which is rich in polyunsaturated fatty acid. Subsequently, a rapid loss of intracellular adenosine triphosphate

Table 2: Effects of antioxidants on sperm motility, sperm count, and sperm morphology

Groups (*n*=5)	Sperm motility (%)	Sperm count ($\times 10^6$/mL)	Sperm morphology (%)
Group A	70.00±5.0	50.83±6.4	20.00±5.3
Group B	95.33±1.5*	68.33±5.1	14.33±1.5
Group C	92.33±2.5*	72.08±5.6*	10.35±1.7*
Group D	82.50±3.5	67.50±3.5	15.50±0.7
Group E	87.50±3.5	87.50±5.3*	15.00±1.4

Values are expressed as mean±SD. *P<0.05 compared with Group A. A - 1 mL of distilled water, B - 400 mg/kg of LC, C - 5 mg/kg of biotin, D - 10 mg/kg of Vit. D, E - LC + biotin + Vit. D, LC - L-carnitine, Vit. D - Vitamin D_3, SD - Standard deviation

from lipid peroxidation causes axonemal damage, decreased sperm viability, and increased mid-piece sperm morphological defects, all of which contribute to decreased sperm motility (Bansal and Bilaspuri, 2010, Agarwal *et al*., 2014). These effects were, however, ameliorated by the antioxidant therapy. Our findings are in concert with Al-Qudah and Ismail (2012), Nikooyeh *et al*. (2014) and Salama *et al*.(2015).

Contrary to the view that orchidopexy remains the only therapy for the treatment of cryptorchidism (Hadziselimovic, 2012), our study though did not include the administration of any exogenous hormone proved that the use of antioxidants upregulated the testosterone synthesis. This is markedly expressed in the group that received the combined therapy. More so, the photomicrographs of the cryptorchid testis treated with antioxidants, especially the combined therapy showed sufficient availability of interstitial cells of Leydig. These cells are the cells responsible for the synthesis of testosterone (Shima *et al*., 2012) which is necessary for the steady flow of spermatogenesis and the maintenance of a rich presence of cells of the germinal epithelium (Heller *et al*., 2016) as seen in this study.

Conclusion

Cryptorchidism disrupts spermatogenesis through the antioxidant-oxidant pathway. The hyper production of free radicals in the testis leads to the death of spermatozoa. Antioxidant therapy of LC, Biotin, and Vit D mitigates the deleterious effects of heat-induced ROS activity, leading to upregulation of testosterone, and subsequent enhancement of spermatogenesis. Contrary to the view that hormonal therapy may serve as first-line therapy in the treatment of cryptorchidism, we are of the opinion that antioxidant therapy may suffice for both the reduction of redox activities and testicular hormonal requirements for spermatogenesis. However, further research needs to be done to compare outcomes with hormonal supplements and surgical treatments.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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