

Full Length Research Paper

Trino IB ameliorates the oxidative stress of cryptorchidism in the rat

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The study examined the effect of Trino IB, a mixture of oleuropein and α -lipoic acid, on the plasma concentration of γ -glutamyl transpeptidase (GGT), malondialdehyde (MDA), sperm count and apoptotic degeneration of testicular germ cells in experimentally induced cryptorchidism in adult male rats. Oral administration of Trino IB (0.5 ml/kg body weight) for 56 days to rats caused significant reduction ($P<0.05$) in the plasma concentration of GGT in the treated groups, but no significant difference ($P=0.0839$) in the plasma MDA concentration was observed. Histologically, Trino IB administration significantly elevated ($P<0.05$) the number of sperm cells in the treated cryptorchid groups compared to the untreated cryptorchid groups. Trino IB alleviated apoptotic degeneration of germ cell in the testes of the treated groups, while there was apoptotic degeneration in the testicular germ cells of the untreated groups. Trino IB alleviated the deleterious effect of oxidative stress in induced cryptorchidism.

Key words: Cryptorchid, oxidative stress, Trino IB, γ -glutamyl transpeptidase, malondialdehyde.

INTRODUCTION

Trino IB is a mixture of two compounds (oleuropein, a polyphenol found in olive oils and alpha lipoic acid, found in kidney, liver, spinach broccoli and potatoes produced by Trinity laboratories. It is classed as an immune modulator which boosts the immune system (personal communication). Trino IB has been found to be effective in reducing the deleterious effects of sodium arsenite on sperm count and motility in rats. The protective effect was found to be better than that of vitamin E, a known antioxidant. The two major constituents of Trino IB mixture are both known to possess antioxidant properties (Haenen and Bast, 1991; Biewenga et al., 1997; Packer et al., 1995, 2001; Manna et al., 1997). It is quite conceivable that the immune boosting effect of Trino-IB may be largely due to the antioxidant properties of the two major constituents.

Cryptorchidism, which is failure of descent of the testis into the scrotum, is generally associated with male inferti-

lity (Chowdhury and Steinberger, 1970). A delay in orchidopexy beyond one or two years of age has been found to lead to a drastic reduction in the number of spermatogonia available for spermatogenesis (Huff et al., 2001). The testicular damage which results from cryptorchidism is thought to be due to excess generation of free radicals by the cryptorchid testis (Ahotupa and Huhtaniemi, 1992; Peltola et al., 1995). The degree of oxidative stress in a cell depends on the concentration of the free radicals which is determined by the balance between their rate of production and their rate of clearance by various antioxidant compounds and enzymes. When free radical generation exceeds the rate of clearance, oxidative stress sets in and this in turn brings about apoptotic cell death. The generation of excess free radicals have been positively correlated with increased rates of apoptotic cell death in other cell types (Dumont et al., 1999; Slater et al., 1995).

Oyewopo and Togun (2005) found that surgically induced cryptorchidism resulted in time dependent reduction in cauda epididymal sperm concentration and motility. Allopurinol has also been found to reduce the

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apoptotic degeneration of testicular cells by inhibiting xanthine oxidase production of free radicals (Argawal et al., 1994; 2003). Melatonin a highly effective endogenous antioxidant free radical scavenger was found to alleviate the deleterious effects of cryptorchidism on metabolic activity of the testes (Saalu et al., 2006). Studies on cryptorchidism have shown that melatonin which possesses hypothermic properties and highly effective anti-oxidant and free radical scavenger properties can alleviate the effect of free radical and protects the tissue from oxidative damage (Reiter, 1993, 1995 and 1998).

Since generation of free radicals occurs in cryptorchid state and has been implicated in the production of the histological changes observed, we sought to determine whether the Trino IB which has immunomodulating and antioxidant effects will alleviate the changes observed biochemically and histologically in experimentally induced cryptorchidism in the rat.

MATERIALS AND METHODS

Animals

Twenty-four six week old male Sprague-Dawley rats were purchased from the animal house, Physiology department, LAUTECH, Ogbomoso. The animals were housed in wire mesh cages under standard environmental conditions. Pelletized growers mash from Bovage feed Nig. Ltd Ogbomoso and water were provided ad libitum.

The animals were pre-treated with Trino IB a preparation made up of oleuropein and alpha-lipoic acid or distilled water for two weeks before the experimental procedure. Trino IB is a product of trinity immuno-efficient laboratories (Trimlab) Ogbomoso. The drug was purchased from the Pre-Degree Science Complex, LAUTECH, Ogbomoso.

Experimental protocol

Twenty-four male Sprague-Dawley rats were weighed and divided into three groups of eight rats in each group as follows:

Group 1: The normal (non cryptorchid) control (NCC) was given distilled water.

Group 2: The cryptorchid control (CC) the rats were rendered unilaterally cryptorchid in the right testes and given distilled water.

Group 3: served as the experimental groups in which rats were rendered unilaterally cryptorchid in the right testes and treated with 0.5 ml Trino IB.

The drug was administered orally using oral canula between the hours of 9:00 and 10:00 am for 56 days (8 weeks) after the surgically induced cryptorchidism.

Induction of cryptorchidism

At the end of three weeks pre-treatment all the animals were rendered unilaterally cryptorchid by the following procedure. Under strict aseptic conditions, the animals were anaesthetised with ketamine (75 mg/kg body weight). The testes were mobilised through a transverse inguinal incision and the gubernacula of the testes severed. The freed testis was pushed back into the abdomen through the internal inguinal ring which was subsequently closed with 2-0 chromic sutures. All the animals subsequently recovered fully. After the cryptorchidism has been induced, the rats in the treated groups were treated with Trino IB for 56 days (8 weeks) after which the

animals were sacrificed by cervical dislocation.

At the end of the experiment, each rat was weighed and sacrificed by cervical dislocation. Blood sample from each rat was collected (by cardiac puncture) into lithium heparinized capillary tubes. The testes of each rat were harvested and preserved in separate formalin bottles. The blood samples were spun using centrifuge at the rate of 3000 revolutions per minute for 15 min. Plasma was collected from each sample and preserved at a very low temperature (-20°C). The blood of each animal was collected by cardiac puncture and the testes were evaluated. The plasma level of γ -glutamyl transferase, malondialdehyde, sperm count, and number of germ cells present in the testes were determined.

The testicular tissue was fixed in 9% formalin solution, embedded in paraffin, and cut into 3 μ m sections. The sections were stained by the haematoxylin-eosin method.

Determination of sperm count

The testes from each rat were carefully exposed and removed. They were trimmed free of the epididymis and adjoining tissues. From each separated epididymis, the cauda part was removed and placed in a beaker containing 1 ml of physiological saline solution. Each section was quickly macerated with a pair of sharp scissors and left for a few minutes to liberate its spermatozoa into the saline solution. Semen drops were placed on the slide and two drops of warm 2.9% sodium citrate were added. The slide was covered with a cover slip and examined sperm count was done under the microscope using improved Neubauer haemocytometer.

Histological processing of testicular tissue

The testis were cut in slabs of about 0.5 cm thick transversely and fixed in Bouin's fluid for a day after which it was transferred to 70% alcohol for dehydration. After six hours it was transferred to 90% alcohol and left overnight. From 90% to 3 changes of absolute alcohol for 1 h each, then into xylene for about 10 h and later transferred into fresh xylene for about 30 min. The tissue was placed in two changes of molten paraffin wax for 20 min each in an oven at 57°C. They were placed vertically in molten paraffin wax inside a metal mould and left overnight to cool and solidify. It was later trimmed and mounted on wooden blocks. Serial sections were cut using rotary microtome at 5 microns. Sections were floated on a water bath to spread out and later picked into albumenized slides and dried on a hot plate at 52°C.

Slides were put in staining rack and placed in staining well containing xylene to dewax, absolute alcohol (2 changes), 70% alcohol and then to water for 5 min after which they were stained with haematoxyline (Hx) for 3 min. Excess Hx was washed off with water and differentiated with 1% acid alcohol. Sections were rinsed in running tap water and left for 5 min for blueing. Sections were stained with 1% eosin and washed off with water. They were dehydrated with 70, 90% and absolute alcohol and cleared in xylene to remove all traces of water. Mountant (a drop) was placed on the surface of slide and covered with a 22 by 22 cover slip (Avwiro et al., 2004). Apoptotic cells were identified by the H and E staining; the number of germ cells in 20 cross sections of the seminiferous tubules was counted.

Enzymes' assay

Plasma γ -glutamyl transferase (GGT) was assayed using a clinical kit (Quinica Clinica Applicada S.A, Spain) based on the method of Szasz (1969). Plasma malondialdehyde (MDA) was determined according to the method of Ohkawa et al. (1979).

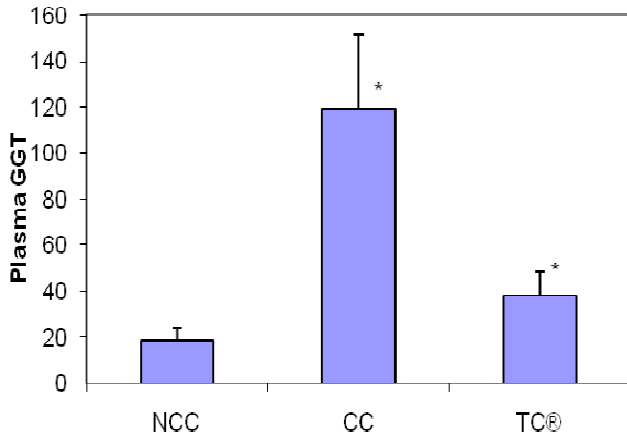


Figure 1. Plasma γ -glutamyl transferase (GGT) in non-cryptorchid (NCC) and cryptorchid control (CC) compared to treated cryptorchid (TC) rats.

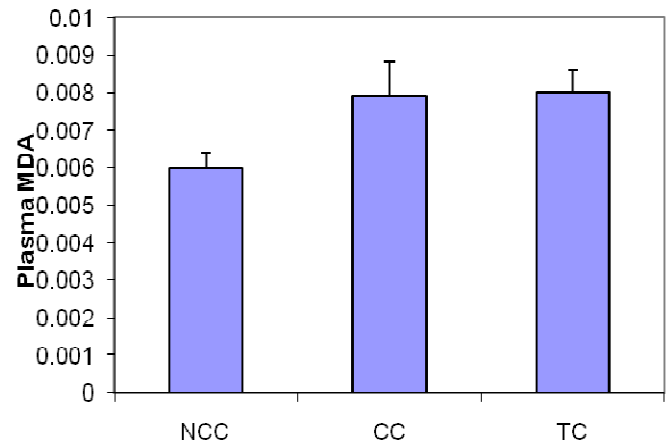


Figure 2. Plasma malondialdehyde (MDA) in non-cryptorchid (NCC) and cryptorchid control (CC) rats in comparison to treated cryptorchid (TC) rats.

Data processing

Data were expressed in mean \pm standard deviation, if applicable. The significance of difference was analyzed using Student's t-test on Microsoft excel and $P < 0.05$ was set as level of significance.

RESULTS AND DISCUSSION

Figure 1 shows the effect of Trino IB on the plasma concentration of γ -glutamyl transferase (GGT) in the treated and untreated groups. The plasma concentration of GGT in the untreated cryptorchid rats (CC) was significantly elevated ($P = 0.0005$) in comparison to the non-cryptorchid (NCC) rats. The plasma concentration of GGT in the treated cryptorchid rats (TC) was significantly depressed ($P = 0.0008$) compared to the untreated cryptorchid rats (CC). Figure 2 shows the effect of Trino IB on mean plasma concentration of malondialdehyde (MDA) in the treated and untreated groups. The plasma concentration of MDA in the treated cryptorchid rats (TC) was not significantly different ($P = 0.0839$) compared to the untreated cryptorchid (CC). The plasma concentration of MDA in the TRCT was also not significantly different ($P = 0.4201$) compared to the URCT. Figure 3 shows the effect of Trino IB on the number of sperm cells present in the cryptorchid testes of the treated and untreated groups. The number of sperm cells in the treated cryptorchid rats (TC) was significantly elevated ($P = 0.0002$) compared to the untreated cryptorchid rats (CC). Figure 4 shows the comparison of the mean values of the number of germ cells present in the evaluated cryptorchid testes of the treated and untreated groups. The mean value of the number of the germ cells present in the TRCT was significantly elevated ($P = 0.0020$) compared to the URCT. The mean value of the number of germ cells present in the TLCT was significantly elevated ($P = 0.0020$) compared to the ULCT. The mean value of the

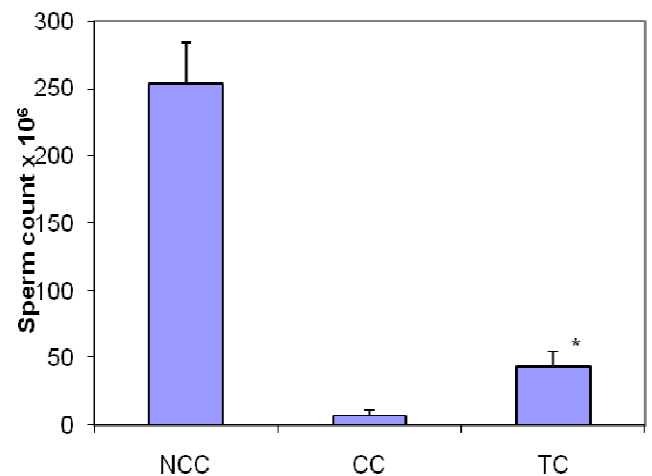


Figure 3. Sperm count in non-cryptorchid (NCC) and cryptorchid control (CC) rats in comparison to treated cryptorchid (TC) rats.

number of germ cells present in the URCT was not significantly different ($P = 0.109$) compared to the ULCT. However, the mean value of the number of germ cells present in the TRCT was significantly lowered ($P = 0.0030$) compared to the TLCT.

The present data show that oxidative stress, disorder of spermatogenesis and apoptotic death of germ cells in experimental cryptorchidism can be prevented by the administration of Trino IB. Trino IB significantly reduced the concentration of the plasma GGT in cryptorchid testes. GGT is a measure of the amount of the enzyme GGT in the blood. An increase in the plasma concentration of GGT may indicate myocardial infarction (Hood et al., 1990), congestive cardiac failure and liver cancer (Stark, 1991). Therefore, it is regarded as a biomarker of oxidative stress and carcinogenesis (Ruppin et al., 1982).

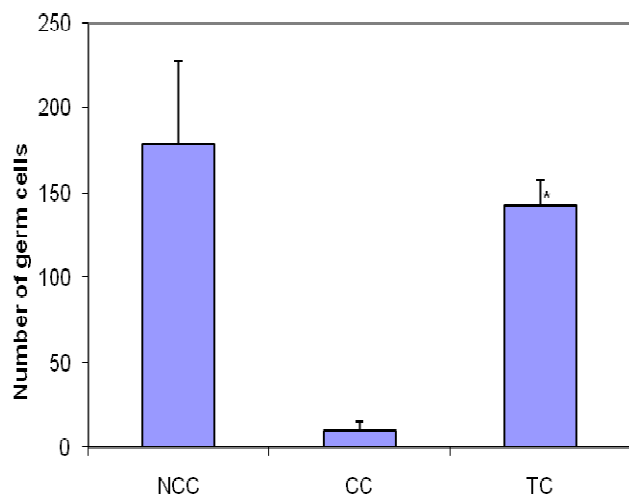


Figure 4. Number of germ cells in non-cryptorchid (NCC) and cryptorchid control (CC) rats in comparison to treated cryptorchid (TC) rats.

Trino IB was able to reduce the plasma concentration of GGT as a result of the antioxidant properties of Oleuropein and alpha lipoic acid. Alpha-lipoic acid has been used to reduce oxidative damage associated with various disease states because it is both water and fat soluble which enhance its ability to reduce oxidation throughout the body (Packer et al., 1995). Also, it has been reported by Berkson et al. (2006), that patients with metastatic pancreatic cancer using alpha lipoic acid have increased long term survival; this is because of alpha-lipoic acid's ability to modify gene expression by stabilizing NFB factor. In addition, it has been found to have activity in breaking peroxidative chain reactions and preventing metal ion chelation, processes that have also been linked to the pathogenesis of heart disease and cancer (Manna et al., 1997), especially prostate and colon (Martin-Moreno et al., 1994). Thus, this may be the mechanism by which Trino IB ameliorates the consequences of cryptorchidism on the plasma concentration of GGT.

The result of the estimation of plasma MDA shows that there was no reduction in the plasma MDA level in all the groups under study. Though, it is expected that the plasma level should be reduced in the treated group when compared with the untreated rather there was an increment. It has been reported that MDA is a stable end product of lipid peroxidation and therefore can be used as an indirect measure of the cumulative lipid peroxidation (Hellstrom et al., 1994; Bell et al., 1992). Although in experimentally induced cryptorchid rats the activities of several scavenging enzymes are impaired, accompanied by increased peroxidation of cellular lipids, the level of lipids peroxidation depends greatly on the rate of ROS production (Ahotupa and Huhtaniemi, 1992). This study examined unilateral cryptorchidism which may account

for the results of the estimation of MDA which shows no indication of lipids peroxidation or no production of MDA unlike in most studies where bilateral cryptorchidism was studied with obvious increase in lipid peroxidation (Yaman et al., 1999). Also, the level of lipid peroxidation in testicular tissue has been previously studied using thiobarbituric acid (TBA) method which was found to be more effective than MDA (Koksal et al., 2000; Koksal et al., 2002).

The low sperm count in the epididymis of the cryptorchid testes of the untreated groups indicates that unilateral cryptorchidism severely impaired spermatogenesis. This is similar to other findings (Ahotupa and Huhtaniemi, 1992). Research during the last decade implicated oxidative stress is a mediator of sperm cells dysfunction (Sharma and Agarwal, 1996; Aitken and Clarkson, 1993). It has been suggested that this phenomenon was causally related to the ability of germ cells to generate reactive oxygen species (ROS). In normal circumstances, there is equilibrium between the generation of ROS and antioxidant strategies of the male reproductive tract, leaving only a critical amount of ROS required for normal sperm function (Griveau and Le Lannou, 1997). Excessive production of ROS, however results in destruction of the antioxidant capacity of spermatozoa and seminal plasma causing oxidative stress which damages spermatozoa membrane and causes infertility (Lewis et al., 1995). The increased sperm concentration in the epididymis of the cryptorchid testes of the treated groups indicated a significant improvement over the untreated group. This shows that Trino IB was able to ameliorate the consequences of cryptorchidism on sperm concentration. This result is similar to the finding that Trino IB ameliorated the effect of sodium arsenite on sperm count and corrects malformation in spermatogenesis (personal communication). Also, oleuropein and alpha-lipoic acid are found to be strong antioxidants and at least effective, if not more than other important dietary antioxidants, such as vitamin C and vitamin E (Kohyama et al., 1997).

The mean value of the number of germ cells presents in the cryptorchid testes of the untreated groups was low compared to the treated groups. Surgical inductions of cryptorchidism in experimental animals causes rapid degeneration of testicular germ cells (Davis and Firlit, 1966), the mechanism of which has been attributed to testicular exposure to the suprascrotal abdominal temperature (Chowdhury and Steinberger, 1970; Blackshaw et al., 1973). The increase in the mean value of the number of germ cells presents in the cryptorchid testes indicates that Trino IB was able to ameliorate the consequences of cryptorchidism and reduce testicular germ cell degeneration. Studies have shown that oleuropein possess strong radical scavenging activity (Visioli et al., 1999) and alpha lipoic acid was also shown to scavenge hydroxyl radicals, singlet oxygen and nitric oxide (Packer et al., 1995). This result is also similar to the findings that the

spleen of the animals treated with sodium arsenite along with either Trino IB or vitamin E appeared normal. This suggests that Trino IB is as effective as Vitamin E which is a known antioxidant (Oloke, 2008).

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