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Taurine reverses endosulfan-induced oxidative stress and apoptosis in adult rat testis

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ABSTRACT

The present study was aimed to investigate the mechanistic aspect of endosulfan toxicity and its protection by taurine in rat testes. Pre-treatment with taurine (100 mg/kg/day) significantly reversed the decrease in testes weight, and the reduction in sperm count, motility, viability and daily sperm production in endosulfan (5 mg/kg/day)-treated rats. Sperm chromatin integrity and epididymal L-carnitine were markedly decreased by endosulfan treatment. Endosulfan significantly decreased the level of serum testosterone and testicular 3β-HSD, 17β-HSD, G6PDH and LDH-X. Sperm $\Delta\psi m$ and mitochondrial cytochrome c content were significantly decreased after endosulfan. Testicular caspases-3, -8 and -9 activities were significantly increased but taurine showed significant protection from endosulfan-induced apoptosis. Oxidative stress was induced by endosulfan treatment as evidenced by increased H_2O_2 level and LPO and decreased the antioxidant enzymes SOD, CAT and GPx activities and GSH content. These alterations were effectively prevented by taurine pre-treatment.

In conclusion, endosulfan decreases rat testes weight, and inhibits spermatogenesis and steroidogenesis. It induces oxidative stress and apoptosis by possible mechanisms of both mitochondria and non-mitochondria pathways. These data provide insight into the mode of action of endosulfan-induced toxicity and the beneficial role provided by taurine to counteract endosulfan-induced oxidative stress and apoptosis in rat testis.

1. Introduction

Endosulfan is a cyclodiene broad spectrum insecticide, which is widely used in many parts of the world on wide variety of crops (Mersie et al., 2003). As a result of its widespread use, it is an environmental contaminant and a public health hazard (Jaiswal et al., 2005). Endosulfan is hazardous to various organs including testes (Ozmen and Mor, 2012). Endosulfan is estrogenic and was reported to induce testicular impairment (Wade et al., 1997). The impairments included decreased sperm count, intratesticular spermatid number, and sperm morphology as well as altered activities of testicular marker enzymes (Sinha et al., 2001).

It was reported that oxidative stress is involved in endosulfan-mediated apoptosis in human peripheral blood mononuclear cells in vitro (Ahmed et al., 2008). Moreover, Takhshid et al. (2012) reported that endosulfan induced oxidative stress and sperm toxicity in rat. Apoptosis is regarded as an active and organized form of cell death, triggered in response to physiologic or pathologic stimuli (Hengartner, 2000). Cell death by apoptosis is a part of normal development and maintenance of homeostasis (Tebourbi et al., 1998; Giannattasio et al., 2002), but is also involved in pathological situations associated with sterility. In the testis, apoptosis is such a common programmed event that 75% of germ cells are reduced by spontaneous apoptosis (Allan et al., 1992). Apoptosis serves several important functions in the testis, a few of which include maintaining appropriate germ cell to Sertoli cells ratio, removing defective germ cells and maintenance of overall quality control in sperm production (Shukla et al., 2012). However, excessive or inadequate apoptosis of testicular cells will result in abnormal spermatogenesis or testicular tumors (Lin et al., 1997).

Apoptosis includes an intrinsic pathway and an extrinsic pathway. In the intrinsic apoptotic pathway, the Bcl-2 family plays a major role, which consists of two functionally distinct groups of proteins, antiapoptotic and proapoptotic proteins. After Bcl-2 separation itself from the outer mitochondrial membrane (Nakai et al., 1993), the cytochrome c is released from the mitochondria into the cytosol where it binds to apoptotic protease-activating factor-1 (Apaf-1). Cytochrome c stimulates the formation of a complex with Apaf-1 and caspase-9 (Liu et al., 1996), which subsequently

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activates the executioner caspases-3, -6, and -7. The active caspases then cause cell apoptosis by cleaving a set of proteins, for example, poly (ADP) ribose polymerase (PARP) (Faraone Mennella, 2011). The extrinsic pathway for apoptosis involves Fas ligand (FASL) stimulation of FAS on target cells, which two recruit Fas-associated death domain (FADD) through shared death domains. The Fas/FADD complex activates initiator caspase-8 and subsequently activates executioner caspases, caspases-3 and -7, which affect apoptosis (Nagata and Golstein, 1995; Lee et al., 1997). Therefore, among the caspases, caspase-3 appears to be a key protease in both of the intrinsic and extrinsic apoptotic pathways, as the main executor which plays a vital role in the whole process of apoptosis (Yao et al., 2012).

Taurine (2-aminoethanesulfonic acid) has been identified as the major free β-amino acid in the male reproductive system (Lobo et al., 2000). Taurine has become an attractive candidate for attenuating various toxin- and drug-induced pathophysiological conditions (Ghosh et al., 2009; Das et al., 2010, 2011) through its antioxidant action (Wang et al., 2008; Zulli, 2011). It may also act as a capacitating agent (Meizel et al., 1980; Meizel, 1985) and as a sperm motility factor (Fraser, 1986; Boatman et al., 1990). It maintains the structural integrity of membrane, regulate calcium transport and modify protein phosphorylation (Zhang et al., 2010). Two sources of taurine could account for the intracellular levels of this amino acid: a biosynthetic route from cysteine and/or the specific uptake from the extracellular space. Extracellular taurine comes from dietary sources or from some tissues in which it is synthesized (e.g. liver) and released into circulation (Huxtable, 1992). However, the testicular toxicity of endosulfan and its mechanism is not fully investigated. In addition, the possible protective effect of taurine needs to be explored. The present study was aimed to investigate the mechanistic aspect of endosulfan toxicity and its protection by taurine in rat testes.

2. Materials and methods

2.1. Reagents

Endosulfan and taurine were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Other reagents were of analytical grade.

2.2. Animals

Adult male Wistar rats (90 days) weighing 170 ± 10 g were housed in clean polypropylene cages and maintained on a 12 h light/dark cycle and a temperature of 20–25 °C with *ad libitum* access to food and water. For 7 days before the experiment, rats were handled daily for 5 min to acclimatize them to human contact and minimize their physiological responses to handling for subsequent protocols (Ma and lightman, 1998). All the experiments with animals were carried out according to the guidelines of the Biochemical and Research Ethical Committee at King Abdulaziz University, Jeddah, Saudi Arabia.

2.3. Experimental protocol

The animals were randomly divided into four groups consisting of six animals each. Group I served as normal control receiving saline vehicle through the experimental period. Group II rats served as drug control group and received taurine (100 mg/kg/day dissolved in normal saline) by oral gavage. Group III treated with endosulfan suspended in olive oil (5 mg/kg/day) by oral gavage. Group IV rats received taurine 24 h prior to the administration of endosulfan. The doses of endosulfan (Uboh et al., 2011) and taurine (Das et al., 2009, 2012) in this study were selected on the basis of previous studies. The experiment was continued for 15 consecutive days.

2.4. Necropsy

Twenty four hours after the last dose, blood samples were collected from the retro-orbital sinus, under ether anesthesia. Samples were centrifuged and supernatant serum was separated from the clot as soon as possible and stored at −80 °C until testosterone and L-carnitine assay. Animals were euthanized and the testes were excised immediately, cleaned from adhering fat and connective tissues and the weights were recorded in g. The cauda epididymides from each animal were used for sperm count and motility and epididymal L-carnitine assay. Sperm from epididymal suspension were used for assay of chromatin integrity and mitochondrial membrane potential ($\Delta\psi m$).

One testis was used for evaluation of daily sperm production (DSP). The other testis was homogenized at 4 °C in RIPA buffer containing 150 mM NaCl, 1 mM EDTA, 10 μg/ml PMSF, 1% Triton X-100 and 20 mM Tris–HCl, pH 7.4 in a glass teflon homogenizer for 10s and centrifuged at 14,000×*g* for 20 min at 4 °C. The cytosol supernatant was removed and used as enzyme source for biochemical assay (Bustamante-Marín et al., 2012). The pellet containing the mitochondria was resuspended in lysis buffer and centrifuged at 10,000×*g* for 10 min. Supernatants (mitochondrial fraction) were used for cytochrome c assay. Protein concentrations were determined using a BCA kit (Pierce, Rockford, USA) that employed bovine serum albumin as a standard.

2.5. Sperm count and motility

Cauda epididymides were dissected out, immediately minced in 5 ml of physiological saline and then incubated at 37 °C for 30 min to allow spermatozoa to leave the epididymal tubules. The percentage of motile sperms was recorded using a phase contrast microscope at a magnification of 400×. Total sperm number was determined by using a Neubauer hemocytometer as previously described (Yokoi et al., 2003). To determine sperm motility, 100 sperms each were observed in three different fields, and classified into motile and non-motile sperms, and the motility was expressed as percentage incidence.

2.6. Sperm viability

A 20 μl of sperm suspension was mixed with an equal volume of 0.05% eosin-Y. After 2 min incubation at room temperature, slides were viewed under the microscope using 400× magnifications. Dead sperms appear pink and live sperms were not stained (Wyrobek et al., 1983). Two hundred sperms were counted for each sample and viability percentage was calculated. Sperm viability was defined as the percentage of intact cells (Eliasson, 1977).

2.7. Daily sperm production (DSP)

Daily sperm production (DSP) was determined in adult rats as previously described (Blazak et al., 1993). The testis was decapsulated and homogenized in 50 ml of ice-cold 0.9% sodium chloride solution containing 0.01% Triton X-100 using a Polytron homogenizer (Sharpe et al., 1995). The homogenate was allowed to settle for 1 min and then was gently mixed, and a 10 ml aliquot was transferred to a glass vial and stored on ice. After thorough mixing of each sample, the number of sperm heads (step 19 spermatid head) in four chambers of Neubauer type hemocytometer was counted under a light microscope with 40× objective. To calculate DSP, the number of spermatids at stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle in which these spermatids are present in the seminiferous epithelium.

2.8. Chromatin integrity

Dried smears were fixed in 96% ethanol and acetic acid (1:1) for 30 min, then hydrolyzed with 0.1 N, HCl for 5 min in 4 °C. The slides were washed with distilled water 3 times for 2 min and stained with 0.05% toluidine blue (TB) for 5 min. Sperms with light blue heads were normal with good DNA integrity, while those with dark heads (purple) were abnormal with poor DNA integrity (Erenpreisa et al., 2003; Tsarev et al., 2009).

2.9. Epididymal L-carnitine

L-Carnitine was determined by a modification of the DTNB method (Pearson et al., 1974) using deproteinized sample solution (Fourie et al., 2001). A 0.1 ml portion of the deproteinized solution was added to 1.2 ml of reaction medium containing 0.1 mol/l Tris–HCl buffer (pH 7.5), AcCOA 0.12 ml (1.2 mg/ml), DTNB 30 μl (2 mg/ml) and CAT 6 μl (1.6 mg/ml). The mixture was incubated at 37 °C for 30 min, then 2 ml of Tris–HCl buffer was added and absorbance was measured at 412 nm. L-Carnitine concentration was assayed using the standard curve method and expressed as μmol/mg protein (protein weight before deproteinization).

2.10. Serum L-carnitine

Serum L-carnitine level was determined as previously described (Hisatomi et al., 2008) after deproteinized with perchloric acid. A mixture of 500 μl of sample solution, 1000 μl of coenzyme buffer, 100 μl of enzyme suspension and 600 μl of distilled water was incubated at room temperature for 10 min and absorbance at 340 nm was measured. Reaction was started by addition of 5 μl of enzyme suspension carnitine acetyl transferase to this mixture. Absorbance at 340 nm was measured at two time points, exactly 30 and 40 min after adding the suspension. Serum L-carnitine was expressed as μM.

2.11. Serum testosterone

Testosterone was measured by use of the Pathozyme Testosterone ELISA kit. Briefly, standards, specimens and controls were dispensed into appropriate wells, followed by testosterone HRP reagent and anti-testosterone reagent, before mixing thoroughly and incubating at 37 °C for 90 min. Wells were then rinsed with de-ionized water and substrate solution was dispensed into each well, gently mixed and incubated for 20 min. The reaction was stopped with the 'stop reagent' and the absorbance recorded at 450 nm (Chen et al., 1991).

2.12. Testicular androgenic enzymes activities

The activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) were measured by the method of Bergmeyer (1974). The reaction mixture in a volume of 2.0 ml contained 100 μmol of sodium pyrophosphate buffer (pH 9.0) and 0.5 μmol cofactor NAD for 3β-HSD and NADPH for 17β-HSD, 0.08 μmol of substrate (dehydroepiandrosterone for 3β-HSD and androstenedione for 17β-HSD) and 100 μl of testicular protein. The reactions were carried out in a quartz cuvette of 1.0 cm path length at 23 ± 1 °C. The absorbance at 340 nm was measured at 20 s intervals for 3 min in a UV–Vis spectrophotometer (UV-1700 Shimadzu, Japan). The enzyme activities were expressed as nmol of NAD converted to NADH/min/mg protein (3β-HSD) or nmol of NADPH converted to NADP/min/ mg protein (17β-HSD).

2.13. Testicular marker enzymes

Glucose-6-phosphate dehydrogenase (G6PDH) was assayed by following the rate of reduction of NADP (Deutsch, 1974). The assay mixture contained 0.1 ml of 3.8 mM of NADP, 0.1 ml of 63 mM $MgCl_2$, 0.1 ml of 33 mM glucose-6-phosphate and 0.5 ml of distilled water. Solutions were mixed, incubated for 5 min at 30 °C and the reaction was started by adding the enzyme sample. The rate of increase in absorbance was recorded for 5 min at 340 nm. Lactate dehydrogenase-X (LDH-X) activity was measured using α-ketovaleric acid as the substrate (Meistrich et al., 1977).

2.14. Mitochondrial membrane potential ($\Delta\psi m$)

The mitochondrial membrane potential ($\Delta\psi m$) was measured using a fluorescent dye rhodamine 123 (Rh123), a cell permeable cationic dye that can accumulate in energized mitochondria based on the highly negative $\Delta\psi m$ (Hong and Liu, 2004). Depolarization of $\Delta\psi m$ results in leakage of Rh123 from mitochondria, and a decrease in intracellular fluorescence. Sperm samples dispersed in physiological saline were adjusted to obtain a final concentration of 10×10^6 sperm/ml and loaded with 10 μM of Rh123 at 37 °C in the dark for 30 min. After being loaded with Rh123, sperm were washed and the fluorescence intensity was assessed using a spectrofluorimeter with excitation wavelength at 488 nm and emission wavelength at 530 nm. The $\Delta\psi m$ was expressed as fluorescence intensity of Rh123.

2.15. Cytochrome c release

Cytochrome c release from sperm cell mitochondria was assayed indirectly by the total mitochondrial cytochrome c content according to Borutaite et al. (1999). Mitochondrial supernatants were used for spectrophotometric measurement of cytochrome c released from mitochondria by using the absorption difference of the wavelengths pair 550/540 nm for ascorbate-reduced minus ferricyanide-oxidized cytochrome c (Rigobello et al., 1999).

2.16. Caspase-3, -8, -9

Enzymatic activities of caspase-3, -8 and -9 was assayed as per the manufacturer's protocol supplied in the caspase colorimetric assay kit. Briefly, an aliquot of enzyme source (100 μg protein/50 μl) was added to 50 μl of reaction buffer containing 200 μM of chromogen (Ac-DEVD-pNA/Ac-IETD-pNA/Ac-LEHD-pNA for caspase-3, -8, -9 respectively), kept at 37 °C for 2 h and terminated by adding stop buffer. The change in the absorbance due to the release of p-nitroanilide (p-NA) was measured at 405 nm using a microtitre plate reader.

2.17. Oxidative stress markers

2.17.1. Hydrogen peroxide (H_2O_2) generation

Hydrogen peroxide (H_2O_2) generation was assayed by the method of Pick and Keisari (1981). Briefly, the incubation mixture contained 1.641 ml phosphate buffer (50 mM, pH7.6), 54 μl horse radish peroxidase (8.5 units/ml), 30 μl of 0.28 nM phenol red, 165 μl of 5.5 nM dextrose, and 600 μl of enzyme source, incubated at 35 °C for 30 min. The reaction was terminated by the addition of 60 μl of 10 N sodium hydroxide. The absorbance was read at 610 nm against a reagent blank on a spectrophotometer. For standard curve, known amounts of hydrogen peroxide and all the above reagents except enzyme source were incubated for 30 min at 35 °C, followed by addition of 60 μl of 10 N sodium hydroxide, and reading of optical density at 610 nm. The quantity of H_2O_2 produced was expressed as nmol of H_2O_2 generated/min/mg protein at 35 °C.

2.17.2. Lipid peroxidation (LPO)

Malondialdehyde (MDA), formed as an end product of the peroxidation of lipids, served as an index of the intensity of oxidative stress. MDA reacts with thiobarbituric acid to generate a colored product that can be measured optically at 532 nm. A break down product of LPO, thiobarbituric acid reactive substance was measured by the method of Buege and Aust (1976). Briefly, the stock solution contained equal volumes of trichloroacetic acid 15% (w/v) in 0.25 N HCl and 2-thiobarbituric acid 0.37% (w/v) in 0.25 N HCl. One volume of the test sample (sperm suspension) and two volumes of stock reagent were mixed in a screw-capped centrifuge tube, vortexed and heated for 15 min on a boiling water bath. After cooling on ice the precipitate was removed by centrifugation at 1000*g* for 15 min and absorbance of the supernatant was measured at 532 nm against blank containing all the reagents except test sample. The value is expressed as μmol of MDA equivalent formed/min/mg protein.

2.18. Assessment of enzymatic antioxidants

Superoxide dismutase (SOD) was assayed by the method of Marklund and Marklund (1974). Briefly, the assay mixture contained 2.4 ml of 50 mM Tris–HCl buffer containing 1 mM EDTA (pH 7.6), 300 μl of 0.2 mM pyrogallol and 300 μl enzyme source. The decrease in absorbance was measured immediately at 420 nm against blank at 10 s intervals for 3 min on a spectrophotometer. The activity of enzyme was expressed in nmol pyrogallol oxidized/min/mg protein. Catalase (CAT) was assayed as previously mentioned (Claiborne, 1985). Briefly, the assay mixture contained 2.40 ml of phosphate buffer (50 mM, pH 7.0), 10 μl of 19 mM H_2O_2 and 50 μl enzyme source (sperm suspension). The decrease in absorbance was measured immediately at 240 nm against blank at 10 s intervals for 3 min on a spectrophotometer. The activity of enzyme was expressed in μmol of H_2O_2 consumed/min/mg protein. Glutathione peroxidase (GPx) was assayed by the method of Paglia and Valentine (1967). Briefly, the assay mixture contained 1.59 ml of phosphate buffer (100 mM, pH 7.6), 100 μl of 10 mM EDTA, 100 μl of sodium azide, 50 μl of glutathione reductase, 100 μl of reduced glutathione, 100 μl of 200 mM NADPH, 10 μl of H_2O_2 and 10 μl enzyme source. The oxidation of NADPH was measured immediately at 340 nm against blank at 10 s intervals for 3 min on a spectrophotometer. The activity of enzyme was expressed in nmol of NADPH oxidized/min/mg protein.

2.19. Assessment of non-enzymatic antioxidant

Total reduced glutathione (GSH) was determined by the method of Moren et al. (1979). Briefly, 1.0 ml of cell extract was precipitated with 1.0 ml of 10% trichloroacetic acid. The precipitate was removed by centrifugation. To an aliquot of the supernatant, 4 ml of phosphate solution and 0.5 ml of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) reagent was added. The color developed was read at 412 nm against a reagent blank. The amount of glutathione was expressed as μg/mg protein.

2.20. Statistical analysis

Differences between obtained values (mean ± SD, $n = 6$) were compared by one way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison test. A P value less than 0.05 was taken as a criterion for a statistically significant difference.

3. Results

There was no mortality, morbidity or distinctive clinical signs observed in any of the experimental groups during the study period.

3.1. Effect of endosulfan and taurine on testes weight and sperm parameters

Table 1 displays the changes in sperm parameters. Treatment of male rats with endosulfan caused significant decrease ($p < 0.01$) in testes weight, sperm count and daily sperm production (Group III) as compared to control animals. Sperm motility and sperm viability were significantly inhibited ($p < 0.001$ & $p < 0.05$ respectively) in response to endosulfan treatment in comparison to control group. Taurine pretreatment (Group IV) reverted the values of sperm parameters to normalcy ($p < 0.05$).

Table 1
Effect of endosulfan and taurine on testes weight and sperm parameters.

Parameter	Group I (control)	Group II (taurine)	Group III (endosulfan)	Group IV (taurine + endosulfan)
Absolute testes weight (g)	2.6 ± 0.19	2.68 ± 0.15	2.2 ± 0.21[a,**]	2.53 ± 0.22[b,*]
Cauda sperm count ($\times 10^6$/rat)	58.5 ± 4.72	59 ± 3.52	49.33 ± 3.39[a,**]	55.67 ± 3.27[b,*]
Sperm motility (%)	87 ± 5.06	88.17 ± 4.62	57.17 ± 4.36[a,***]	83.5 ± 2.59[b,*]
Sperm viability (%)	90.5 ± 4.68	91.67 ± 5	81 ± 7.07[a,*]	89.67 ± 4.03[b,*]
Daily sperm production (10^6/g testis/day)	29.33 ± 3.78	29.5 ± 3.94	22.5 ± 2.88[a,**]	28 ± 2.45[b,*]

Data are expressed as mean ± S.D. ($n = 6$). Statistical analysis (ANOVA) for differences from corresponding control.
[a] Comparisons were made between: Group I and Groups II–IV.
[b] Comparisons were made between: Group III and Group IV.
[*] Statistical significance from control where $p < 0.05$.
[**] Statistical significance from control where $p < 0.01$.
[***] Statistical significance from control where $p < 0.001$.

3.2. Effect of endosulfan and taurine on epididymal sperm chromatin integrity

The effect of endosulfan on chromatin integrity of epididymal sperm and the protection rendered by taurine is presented in Fig. 1. The decrease in chromatin integrity in endosulfan-treated rats (Group III) was evident from increased percentage ($p < 0.05$) of positive toluidine blue staining in the epididymal sperm. This increased percentage is significantly different from the related control. Taurine pretreatment (Group IV) significantly ($p < 0.01$) suppressed the change in chromatin quality induced by endosulfan.

3.3. Effect of endosulfan and taurine on epididymal and serum L-carnitine

Table 2 presents the significant ($p < 0.05$) decrease in epididymal L-carnitine in response to endosulfan treatment (Group III) as compared to the corresponding control. However, taurine pretreatment (Group IV) restored L-carnitine content toward normalcy. Serum L-carnitine did not show any significant difference in response to endosulfan treatment as compared to the corresponding control (Table 2).

3.4. Effect of endosulfan and taurine on serum testosterone and testicular steroidogenic enzymes

Serum testosterone was significantly ($p < 0.001$) decreased in response to endosulfan treatment (Group III) (Table 3) as compared to the corresponding control. Taurine pretreatment (Group IV) maintained serum testosterone concentration ($p < 0.001$) near normal amount. The activities of 3β-HSD and 17β-HSD were significantly ($p < 0.01$) inhibited in response to endusulfan treatment

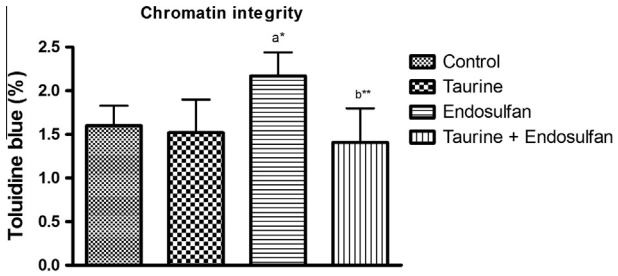

Fig. 1. Effect of endosulfan and taurine treatment on rat sperm chromatin integrity. Group I: Control, Group II: Taurine, Group III: Endosulfan, Group IV: Taurine + Endosulfan. Data are expressed as mean ± S.D. ($n = 6$). Statistical analysis (ANOVA) for differences from corresponding control: Comparisons were made between: [a]Group I and Groups II–IV; [b]Group III and Group IV. The symbols represent statistical significance from control where $^{*}p < 0.05$; $^{**}p < 0.01$.

(Group III) as compared to the related control (Table 3). Pretreatment with taurine significantly ($p < 0.05$) prevented these alterations induced by endosulfan (Group IV).

3.5. Effect of endosulfan and taurine on testicular G6PDH and LDH-X enzymes activities and sperm $\Delta\psi m$

The activities of G6PDH and LDH-X enzymes reveal a significant decrease (13.11% and 28.16% respectively) (Group III) as compared to the related control. The activities of these enzymes were normalized by comparison to the taurine pretreatment (Group IV, Fig. 2A and B respectively). The $\Delta\psi m$ was significantly decreased (28.16%) in response to endosulfan treatment (Group III) and was normalized by taurine pretreatment (Group IV, Fig. 3).

3.6. Effect of endosulfan and taurine on mitochondrial cytochrome c content and testicular caspases

Table 4 demonstrates the changes in mitochondria cytochrome c content and caspases-3, -8 and -9 in response to endosulfan treatment. Endosulfan caused significant ($p < 0.01$) decrease in mitochondrial cytochrome c content (Group III). Pretreatment with taurine could rescue this translocation effectively ($p < 0.05$) (Group IV). Caspases-3, -8 and -9 activities were significantly ($p < 0.01$, $p < 0.05$ and $p < 0.05$ respectively) increased in response to endosulfan treatment (Group III). This endosulfan-induced increase in caspases activities was absent in animals pretreated with taurine (Group IV).

3.7. Effect of endosulfan and taurine on oxidative stress parameters

Markers of testicular tissue lipid peroxidation and antioxidant enzymes of all the groups are presented in Table 5. Hydrogen peroxide (H_2O_2) level and lipid peroxidation (LPO) were significantly ($p < 0.01$) (28.3% and 25% respectively) increased in response to endosulfan treatment (Group III). Taurine pretreatment (Group IV) prevented these abnormalities. Endosulfan-treatment (Group III) decreased the specific activities of SOD (20.96%), CAT (18.1%) and GPX (17.34%) in comparison to the corresponding control (Group I). Total reduced GSH was significantly ($p < 0.001$) decreased (25.09%) in response to endosulfan treatment (Group III) as compared to non-treated animals. All the endosulfan-induced alterations in the antioxidant enzymatic activities were effectively prevented by taurine pretreatment (Table 5).

4. Discussion

In the present study, reduction in the testes weight after endosulfan treatment was indicative of toxicity. Because the weight of the testes largely depend on the mass of the differentiated

Table 2
Effect of endosulfan and taurine on epididymal and serum L-carnitine.

Parameter	Group I (control)	Group II (taurine)	Group III (endosulfan)	Group IV (taurine + endosulfan)
Epididymal L-carnitine (μmol/mg protein)	0.68 ± 0.037	0.69 ± 0.028	0.61 ± 0.043[a,*]	0.67 ± 0.032[b,*]
Serum L-carnitine (μM)	41 ± 2.37	42.17 ± 3.71	43.17 ± 3.43	42.5 ± 2.43

Data are expressed as mean ± S.D. ($n = 6$). Statistical analysis (ANOVA) for differences from corresponding control.
[a] Comparisons were made between: Group I and Groups II–IV.
[b] Comparisons were made between: Group III and Group IV.
[*] Statistical significance from control where $p < 0.05$.

Table 3
Effect of endosulfan and taurine on serum testosterone and testicular steroidogenic enzymes.

Parameter	Group I (control)	Group II (taurine)	Group III (endosulfan)	Group IV (taurine + endosulfan)
Serum testosterone (ng/ml)	4.63 ± 0.4	4.72 ± 0.57	3.58 ± 0.15[a,***]	4.62 ± 0.31[b,***]
3β-HSD	40.17 ± 3.37	41 ± 3.74	31.5 ± 3.99[a,**]	38.5 ± 3.39[b,*]
17β-HSD	32.33 ± 3.56	32.83 ± 3.25	25 ± 2.53[a,**]	30.67 ± 3.14[b,*]

Data are expressed as mean ± S.D. (n = 6). Statistical analysis (ANOVA) for differences from corresponding control. 3β-HSD: 3β-hydroxysteroid dehydrogenase, nmol of NAD converted to NADH/min/mg protein; 17β-HSD: 17β-hydroxysteroid dehydrogenase, nmol of NADPH converted to NADP/min/mg protein.
[a] Comparisons were made between: Group I and Groups II–IV.
[b] Comparisons were made between: Group III and Group IV.
[*] Statistical significance from control where $p < 0.05$.
[**] Statistical significance from control where $p < 0.01$.
[***] Statistical significance from control where $p < 0.001$.

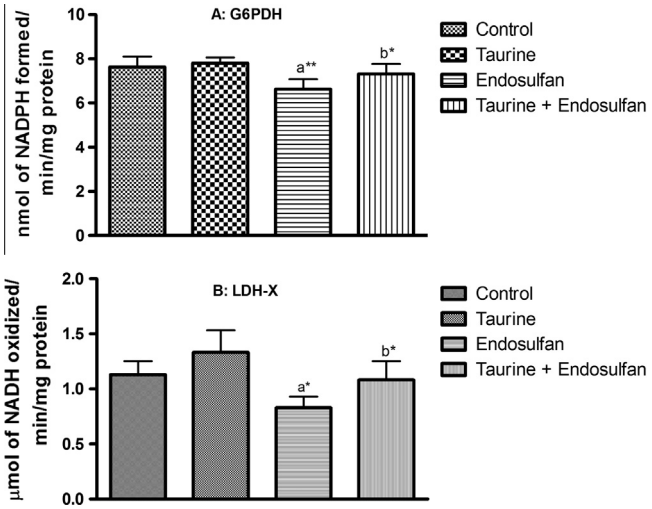

Fig. 2. Effect of endosulfan and taurine treatment on rat testicular glucose-6-phosphate dehydrogenase (G6PDH) (A) and lactate dehydrogenase-X (LDH-X) (B) activities. Group I: Control, Group II: Taurine, Group III: Endosulfan, Group IV: Taurine + Endosulfan. Data are expressed as mean ± S.D. ($n = 6$). Statistical analysis (ANOVA) for differences from corresponding control: comparisons were made between: [a]Group I and Groups II–IV; [b]Group III and Group IV. The symbols represent statistical significance from control where $^{*}p < 0.05$; $^{**}p < 0.01$.

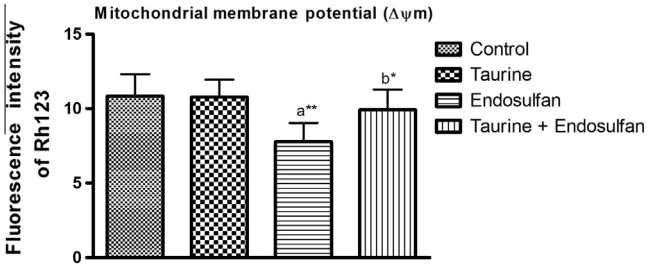

Fig. 3. Effect of endosulfan and taurine treatment on rat sperm mitochondrial membrane potential ($\Delta\psi m$). Group I: Control, Group II: Taurine, Group III: Endosulfan, Group IV: Taurine + Endosulfan. Data are expressed as mean ± S.D. ($n = 6$). Statistical analysis (ANOVA) for differences from corresponding control: comparisons were made between: [a]Group I and Groups II–IV; [b]Group III and Group IV. The symbols represent statistical significance from control where $^{*}p < 0.05$; $^{**}p < 0.01$.

spermatogenic cells, a significant decline in the weight of testes, an excellent indicator of gonadal toxicity, would be expected which may be due to the marked decrease in testosterone production, significant lower rate of spermatogenesis and decreased sperm production (Katoh et al., 2002; Prahalathan et al., 2004; Pandya et al., 2012). Taurine pretreatment protected these animals from retardation in testicular growth. The decreased sperm count suggests that endosulfan affects spermatogenesis (Kao et al., 2008). The decrease in sperm count in endosulfan-treated rats may be due to the lower concentration of testosterone as the sperm production in testis and maturation in epididymis is under the control of testosterone (Sharpe et al., 1992). Sperm motility is also as important as the counts in respect to the fertility in the male. The significant reduction in sperm motility may be due to the toxic effect of endosulfan on the flagellum, the important machinery for motility and/or on sperm cells. Further, the decreased sperm count, motility and viability points to lipid peroxidation and reactive oxygen species production (ROS) generated (Kao et al., 2008). In addition to decrease in sperm count, motility and viability, the toxicity of endosulfan was further justified by a decrease in daily sperm production manifested as decrease in step 19 spermatids/stage VII (D'Souza and Narayana, 2002). The reduction in daily sperm production of the rats treated with endosulfan, was consistent with the reduction in concentrations of serum testosterone. It is suggested that endosulfan may decrease daily sperm production by disturbing testosterone biosynthesis. These alterations were effectively prevented by taurine pre-treatment.

Normally, sperm chromatin is a highly organized and compact structure consisting of DNA and heterogeneous nucleoproteins (Lolis et al., 1996). Sperm DNA integrity is essential for the accurate transmission of genetic information. Oxidative stress and poor chromatin packaging can affect the integrity of sperm chromatin and cause sperm DNA damage (Lakpour et al., 2008). Any form of sperm chromatin abnormalities or DNA damage may result in male infertility (Cocuzza et al., 2007). According to one of the most important hypotheses, the molecular mechanism of sperm DNA

Table 4
Effect of endosulfan and taurine on mitochondrial cytochrome c content and testicular caspases.

Parameter	Group I (control)	Group II (taurine)	Group III (endosulfan)	Group IV (taurine + endosulfan)
Mitochondrial cytochrome c content (nmol/mg protein)	0.29 ± 0.036	0.3 ± 0.016	0.23 ± 0.028[a,**]	0.28 ± 0.015[b,*]
Caspase-3 (Abs)	0.1 ± 0.014	0.09 ± 0.012	0.15 ± 0.03[a,**]	0.11 ± 0.018[b,*]
Caspase-8 (Abs)	0.12 ± 0.016	0.12 ± 0.019	0.15 ± 0.015[a,*]	0.13 ± 0.01[b,*]
Caspase-9 (Abs)	0.09 ± 0.012	0.09 ± 0.015	0.14 ± 0.028[a,*]	0.12 ± 0.016[b,**]

Data are expressed as mean ± S.D. ($n = 6$). Statistical analysis (ANOVA) for differences from corresponding control.
[a] Comparisons were made between: Group I and Groups II–IV.
[b] Comparisons were made between: Group III and Group IV.
[*] Statistical significance from control where $p < 0.05$.
[**] Statistical significance from control where $p < 0.01$.

Table 5
Effect of endosulfan and taurine on oxidative stress status.

Parameter	Group I (control)	Group II (taurine)	Group III (endosulfan)	Group IV (taurine + endosulfan)
H_2O_2 production	27.67 ± 3.61	26.17 ± 2.93	35.5 ± 4.18[a,**]	30 ± 2.28[b,*]
LPO	3.12 ± 0.35	3 ± 0.32	3.9 ± 0.46[a,**]	3.22 ± 0.32[b,*]
SOD	24.67 ± 2.94	25 ± 2.37	19.5 ± 2.43[a,*]	23.83 ± 2.32[b,*]
CAT	3.15 ± 0.31	3.22 ± 0.33	2.58 ± 0.25[a,*]	3.07 ± 0.25[b,*]
GPx	57.67 ± 4.76	58.33 ± 5.2	47.67 ± 3.88[a,**]	56.33 ± 2.73[b,*]
GSH	8.05 ± 0.83	8.85 ± 1.03	6.03 ± 0.53[a,***]	7.43 ± 0.45[b,*]

Data are expressed as mean ± S.D. (n = 6). Statistical analysis (ANOVA) for differences from corresponding control. H_2O_2: Hydrogen peroxide, nmol of H_2O_2 generated/min/mg protein; LPO: Lipid peroxidation, μmol of malondialdehyde equivalent formed/min/mg protein; SOD: Superoxide dismutase, noml of pyrogallol oxidized/min/mg protein; CAT: Catalase, μmol of H_2O_2 consumed/min/mg protein; GPx: Glutathione peroxidase, nmol of NADPH oxidized/min/mg protein; GSH: Total reduced glutathione, μg/mg protein.
[a] Comparisons were made between: Group I and Groups II–IV.
[b] Comparisons were made between: Group III and Group IV.
[*] Statistical significance from control where $p < 0.05$.
[**] Statistical significance from control where $p < 0.01$.
[***] Statistical significance from control where $p < 0.001$.

damage involves ROS (Agarwal and Said, 2003; Cocuzza et al., 2008) which causes peroxidation of sperm plasma membrane and nuclear DNA damage (Enciso et al., 2006). Toluidine blue test is suggested to be a simple alternative for other sperm chromatin assessment tests like TUNEL and sperm chromatin structural assay (Talebi et al., 2012). The present study demonstrated that a higher percentage of sperm with fragmented DNA was observed in animals treated with endosulfan than the control. The positive changes observed in sperm chromatin integrity in this study may be attributed to the antioxidant effect of taurine. Taurine directly scavenges hydroxyl radicals and inhibits DNA damage. Perhaps, the interference of taurine with free radical generation could be one of the causes of increased DNA repair in endosulfan-treated animals.

It was reported that the level of epididymal L-carnitine is a biochemical marker of epididymal function (Hisatomi et al., 2008) and sperm count, motility and maturation are related to epididymal L-carnitine concentration (Ng et al., 2004). It is suggested that the decrease in epididymal L-carnitine contributes directly to decrease sperm count, motility and viability. Since L-carnitine exists also in the sperm, the decrease in L-carnitine in the epididymis could be assumed due to the decrease in sperm count, or in L-carnitine content in the sperm itself. However, L-carnitine content in the epididymal tissue fluid is much greater than that in the sperm (Cooper et al., 1997), it was considered that a large part of this decrease in L-carnitine reflected a decrease in its content in the epididymal fluid. Among the parameters examined including serum L-carnitine, only a decrease in L-carnitine level in the cauda epididymis was detected. This suggests that lowering of L-carnitine in the cauda epididymis is attributable to the adverse effect on epididymal function to transport and/or concentrate L-carnitine. The decrease in sperm count and in L-carnitine level in the epididymis appeared to be in parallel. These alterations returned to normalcy by taurine pretreatment.

Testosterone is required for the attachment of different generations of germ cells in seminiferous tubules and therefore low level of testicular testosterone may lead to detachment of germ cells from seminiferous epithelium and may initiate germ cell apoptosis (Blanco-Rodríguez and Martínez-García, 1998). Leydig cells synthesize testosterone and secrete it into the blood stream in males (Hu et al., 2011). So, the decreased serum testosterone in the endosulfan-treated rats may be due to Leydig cell impairment caused by ROS generation (Cao et al., 2004; Chen et al., 2008) or due to inhibition of testicular steroidogenic enzymes activity, because these enzymes are responsible for the regulation of testosterone biosynthesis (Jana et al., 2005). The decrease in serum testosterone is accompanied by significant decrease in 3β-HSD and 17β-HSD activities. The testicular steroidogenic enzymes 3β-HSD and 17β-HSDs are the key enzymes in testosterone biosynthesis (Wylie et al., 2010). The decreased steroidogenic enzyme activity levels may lead to decreased steroidogenesis in rat. Therefore, the decrease in the plasma level of testosterone in the rat exposed to endosulfan may be due to the decreased steroidogenesis (Reddy et al., 2011). The reduction in testicular androgenic enzyme activities and decreased serum testosterone may be due to elevated levels of reactive oxygen species and lipid peroxidation (Murugesan et al., 2008). Moreover Ozmen and Mor (2012) reported that the decreased testosterone production may be due to testicular degeneration and apoptotic activity of endosulfan in Leydig cells. Pretreatment with taurine could normalize serum testosterone and testicular steroidogenic enzymes activities.

The present findings also show decreased activities of G6PDH and LDH-X in endosulfan-exposed rats, which are indicative of testicular toxicity. G6PDH is a key enzyme of the testicular tissue (Prasad et al., 1995) and provides reducing equivalents for the hydroxylation of steroids. G6PDH is also directly associated with glutathione metabolism and lowered activity of this enzyme provokes increased oxidative stress to the tissue and may lead to cell

death (Das et al., 2012). LDH-X enzyme is a marker for normal spermatozoa metabolism (Khanna et al., 2011). LDH-X, a unique isoenzyme of lactate dehydrogenase, in inner mitochondrial membrane of spermatogenic cells of mature and developing testis, plays an important role in transferring hydrogen from cytoplasm to mitochondria by redox coupling α-hydroxy acid/α-keto acid related to spermatozoal metabolism (Gu et al., 1989). This could be one of the contributory factors leading to reduced male sperm concentration and sperm motility on endosulfan exposure (Benoff et al., 2009). Taurine could, however, effectively suppress the endosulfan-induced up-regulation alterations in the activities of these enzymes.

Under normal conditions, ROS is mainly generated in mitochondria and is rapidly scavenged by cellular antioxidants (Balaban et al., 2005). In the present study, endosulfan induced mitochondrial membrane depolarization resulting in further increase of ROS generation (Khanna et al., 2011). ROS acts as a critical mediator of apoptosis. Moreover, the decrease in $\Delta\psi m$ is an early marker of apoptosis (Khanna et al., 2011). The present study indicated that treatment with endosulfan induced increase in ROS and decrease in $\Delta\psi m$, which promoted apoptosis mitochondria-mediated pathway.

$\Delta\psi m$ depolarization is followed by cytochrome c release from the mitochondria into the cytosol. This leads to activation of caspase-9 (Antonsson, 2001), which in turn activates caspase-3. Caspase-3 is a prevalent caspase that is ultimately responsible for the majority of apoptotic processes (Tang et al., 2009). After treatment with endosulfan, the activities of caspases-9 and caspases-3 were increased. Activation of caspase-8 initiates a cascade process which in turn activates caspase-9 (Li and Lim, 2007). Ultimately, caspase-3 is activated by activated caspase-9 or caspase-8 and it induces apoptosis (Denecker et al., 2001; Tafani et al., 2002). It is generally assumed that endosulfan induced apoptosis in the rat testis by activation of caspase-8 and caspase-9. Taurine pretreatment, however, effectively suppressed the activation of caspase-3 as well as the release of cytochrome c and enhanced mitochondrial transmembrane potential ($\Delta\psi m$).

Oxidative stress is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen-derived oxidants commonly known as ROS (Sikka, 1996). Oxidative damage induced by ROS is implicated as an important contributing factor in male infertility. An excessive production of ROS may result in an impaired steroidogenesis (Diemer et al., 2003) and spermatogenesis (Sen Gupta et al., 2004). These ROS include oxygen free radicals such as superoxide, hydroxyl, peroxyl, alkoxyl and hydroperoxyl radicals which cause tissue damage by a variety of mechanisms including DNA damage, lipid peroxidation, protein oxidation and depletion of thiols. The present findings showed that endosulfan exposure had a marked oxidative impact in rat testis as evidenced by the significant increase in H_2O_2 production and LPO. Because of high concentration of polyunsaturated fatty acids and low antioxidant capacity, male germ cells could be susceptible to oxidative stress (Vernet et al., 2004). LPO, a type of oxidative degradation of polyunsaturated fatty acids, has been linked with altered membrane structure and enzyme inactivation (Selvakumar et al., 2004). These changes might result from increased production of free radicals and/or a decrease in antioxidant status. The present study also shows that the changes in LPO are accompanied by concomitant decrease in the activities of antioxidant enzymes, namely SOD, CAT and GPX, as well as the levels of reduced glutathione. SOD constitutes an important link in the biological defense mechanism through dismutation of endogenous cytotoxic superoxide radicals to H_2O_2 and molecular oxygen that are deleterious to polyunsaturated fatty acids and proteins (Fridovich, 1975). The reduction in the activities of CAT and GPx may reflect the inability of testicular cells to eliminate the H_2O_2 produced by SOD. This may also be attributed to enzyme inactivation caused by excess ROS production (Pigeolet et al., 1990). The antioxidant enzymes CAT and GPx protect SOD against inactivation by H_2O_2. Reciprocally, SOD protects CAT and GPx against superoxide anion. Thus, the balance of this enzyme system is essential to dispose the superoxide anion and peroxides generated in the testis (Prahalathan et al., 2006). The reduction in the activities of these enzymes and increase in LPO could reflect the adverse effect of endosulfan on the antioxidant system in testis. GSH plays an important role in the detoxification of xenobiotics and in the antioxidation of ROS and free radicals. It is important in the regulation of the cellular redox state and a decline in its cellular level has been considered to be indicative of oxidative stress (Bray and Taylor, 1993). Taurine treatment reversed all these adverse effects and oxidative stress-associated changes induced by endosulfan. This beneficial effect of taurine as an antioxidant in organ pathophysiology has been attributed to its ability by stabilizing biological membrane and scavenging reactive oxygen species (Cozzi et al., 1995). Based on the results of earlier investigations, taurine could be proposed to play its protective role by its direct as well as indirect antioxidant activities. As a direct antioxidant, it could quench and detoxify several reactive intermediates, like nitric oxide (Redmond et al., 1996), H_2O_2 (Cozzi et al., 1995), hydroxyl radical (·OH) (Aruoma et al., 1988), etc. As an indirect antioxidant, it could prevent the changes in oxidative stress-induced membrane permeability and stabilize it (Timbrell et al., 1995; Gordon and Heller, 1992).

The present data indicated that endosulfan could induce apoptosis in rat testis via mitochondrial and non-mitochondrial pathways via decrease in $\Delta\psi m$ along with the cytochrome c release from mitochondria into the cytosol and activation of caspases-3, -8, and -9. These apoptotic changes might elucidate the data of the present study that endosulfan decreased testes weight, sperm count, motility and viability, in addition to daily sperm production. Endosulfan decreased sperm chromatin integrity, epididymal L-carnitine, serum testosterone, and testicular 3β-HSD, 17β-HSD, G6PDH and LDH-X activities. Furthermore, endosulfan induced H_2O_2 production, LPO and decreased SOD, CAT and GPx activities and decreased GSH content. Importantly, taurine pretreatment attenuated testicular dysfunction and apoptotic changes. Taurine, as an antioxidant, renewed the activities of the antioxidant enzymes and GSH level, down-regulated the levels of ROS.

In conclusion, endosulfan decreases rat testes weight, and inhibits spermatogenesis and steroidogenesis. It induces oxidative stress and apoptosis by possible mechanisms of both mitochondria and non-mitochondria pathways. These data provide insight into the mode of action of endosulfan-induced toxicity and the beneficial role provided by taurine to counteract endosulfan-induced oxidative stress and apoptosis in rat testis and to restore the suppressed spermatogenesis, thereby proving to be an effective cytoprotectant.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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