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Protective effects of Vitamin E on CCl_4-induced testicular toxicity in male rats

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The increased generation of free radicals plays an important role in testicular damage. The present study aimed to investigate the adverse effects of carbon tetrachloride (CCl_4) on the reproductive system of male rats as well as to examine whether Vitamin E (VE) is able to ameliorate these effects. The rats were equally divided into three groups: control, CCl_4-treated, and CCl_4 + VE-treated groups. After 4 weeks of treatment, the decrease in body and testes weights, sperm parameters, and the decrease in serum levels of testosterone, luteinizing hormone, and follicle-stimulating hormone of CCl_4-treated rats were ameliorated by VE treatment. The co-administration of VE with CCl_4 significantly decreased the level of lipid peroxidation production (malondialdehyde) and increased the activity of anti-oxidant enzymes (superoxide dismutase and catalase) when compared with the CCl_4 group. Moreover, VE prevented CCl_4-induced severe testicular histopathological lesions and deformities in spermatogenesis. The results demonstrate that VE augments the anti-oxidants' defense mechanism against CCl_4-induced reproductive toxicity suggesting a therapeutic role in free radical-mediated infertility.

Keywords: Vitamin E, CCl_4, lipoperoxidation, anti-oxidants, infertility, toxicity, LH, FSH, testosterone

Introduction

Testicular oxidative stress appears to be a common feature in male infertility, which occurs due to deficient sperm production and hormonal imbalance. Spermatogenesis is orchestrated by two main regulations: endocrine [luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from pituitary gland] and local intercellular communications mediated by a complex interplay of hormones and testicular growth factors, among which testosterone is crucial for testicular functioning (3). Recent studies concluded that oxidative stress, exhibited by exposure to toxic chemicals, causes a major damage to sperm quality by disrupting the anti-oxidant and reactive oxygen species (ROS) balance and thus resulting in abnormalities of spermatogenesis and male infertility (22, 32).

Carbon tetrachloride (CCl_4) exerts its toxic/hazardous effects via the production of free radicals. The free radicals generated from CCl_4 upon the activation of liver cytochromes P450 react directly with cell membranes. These free radicals not only target the liver but can also cause free-radical production in other tissues including lung, heart, kidneys, testes, brain, and blood (21). Free radicals produced from CCl_4 metabolism can generate highly reactive lipid peroxides via binding to polyunsaturated fatty acid (PUFA) of spermatozoon membrane and alter sperm

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concentration, hormonal levels, enzymatic activity, and can induce necrosis. Spermatozoa are rich in PUFA, and therefore, could be highly susceptible to oxidative stress (11).

The most important anti-oxidant enzymes include glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD), which play the key roles in detoxification of oxygen free radicals (38). SOD catalyzes the superoxide anion into oxygen and hydrogen peroxide, which are later on converted into water by CAT (39). ROS also affects the anti-oxidant defense mechanism by reducing the intracellular concentration of GPx and decreasing the activity of SOD and CAT (36). When this defense mechanism is not complete or when ROS are excessively generated, additional protecting mechanisms of dietary anti-oxidants could be of a great significance. This explains the increased number of the proposed natural and synthetic anti-oxidative agents that treat reproductive hormonal imbalance and infertility induced by oxidative stress (35). Furthermore, recent results suggested that natural products containing anti-oxidants have the ability to protect testis against oxidative damages, lipid peroxidation, and impairment in anti-oxidant status induced by CCl_4 (3, 13, 35).

Vitamin E (VE) is one of the most potent lipid-soluble free radical scavengers, believed to be one of the primary components of the anti-oxidant system of the spermatozoa, that limit lipid peroxidation in the testicular microsomes and mitochondria (12). Traditionally, VE is called anti-sterility vitamin and is associated with normal functions of male reproductive system, vital for the maintenance of mammalian spermatogenesis, and reverses the detrimental effects of oxidative stress on testicular function mediated by different agents (16, 43). However, *in vivo* testicular histopathological alterations due to CCl_4 intoxication and the protective role of VE seem to be lacking in the literature. To the best of the authors' knowledge, no study has examined the effect of VE on CCl_4-mediated toxicity in epididymal sperm and on hormonal levels of rats. Therefore, the present study was performed to evaluate the possible protective effect of VE against testicular toxicity induced by CCl_4 in male rats.

Materials and Methods

Animals

About 50 healthy adult male albino rats (*Rattus norvegicus*) (120–130 g) were obtained from the animal house of Medical Research Institute, Alexandria University, Egypt. Rats were kept in the animal house under the standard conditions of 21 ± 2 °C with an alternating 12-h light/dark schedule and were allowed to free access to food and water. Rats were acclimated to laboratory conditions for 2 weeks prior to experiments. All animal experiments were conducted in accordance with the Canadian Council on Animal Care (CCAC) guidelines and the Animal Ethics Committee of the Alexandria University, Egypt.

Experimental design

To study the anti-oxidant attributes of VE, male albino rats were equally divided into five groups ($N = 10$). Group 1 (control group): rats received intraperitoneally (i.p.) physiological saline solution 0.75 ml/kg, 4 days/week for 4 weeks. Group 2 (CCl_4-treated group): rats received (i.p.) 3-ml/kg body weight of CCl_4 once a week for 4 weeks (21). CCl_4 were purchased from Loba Company (India). Group 3 (CCl_4 + VE-treated group): rats were treated with the same dose of CCl_4 and 100-mg/kg body weight of VE orally by gavage, 4 days/week for 4 weeks (27). VE was purchased from Sigma Chemical Company (MO, USA). Group 4 (VE-treated group): rats received the same oral dose of VE by gavage for 4 days/week for

4 weeks. Group 5 (OL-treated group): rats received orally olive oil (OL) by gavage at a dose of 0.5-ml/kg body weight, 4 days/week for 4 weeks (33). After 24 h of the last treatment, all animals were weighed, sacrificed by cervical dislocation, and blood samples were collected. Serum samples were separated and stored at −20 °C until the use for hormonal assays.

The testes and epididymis were dissected out, trimmed off the attached tissues, and weighed. One testis of each rat was fixed for histological study and the other testis for biochemical estimation as described in the following. Sperms were collected from the epididymis, mounted on a slide, and then motility assessed immediately under the microscope at 10× objective. The motility assessment was expressed as percentage motile forms. The slides were later stained with Carbol Fuchsin and the sperm number and viability were examined.

Biochemical analysis

Testes were homogenized with three volumes of ice-cold 1.15% KCI. The levels of malondialdehyde (MDA), SOD, and CAT were measured in the supernatant obtained from centrifugation at 14,000 rpm. MDA is the most abundant individual aldehyde resulting from lipid peroxidation breakdown in biological systems, and it is used as an indirect index of lipid peroxidation. Determination of MDA that is based on its interaction with thiobarbituric acid (TBA) to form a pink complex with absorption maximum at 535 nm was assayed, as described previously (18). The activity of the SOD enzyme in the testes homogenate was determined, as described previously (25). The reaction was carried out in 0.05-M sodium carbonate buffer pH 10.3 and was initiated by the addition of epinephrine in 0.005 N HCl. CAT activities were determined, as described previously (1). Absorbance was recorded using Shimadzu Recording Spectrophotometer (UV 160) in all measurements.

Hormonal assay

Serum levels of testosterone, LH, and FSH were estimated using enzyme-linked immunosorbent assay (ELISA) kits (Diagnostic System Laboratories Inc., USA), according to the manufacturer's instruction.

Histopathology

For the histological examinations, testes were fixed in 10% buffered formalin for 24 h at room temperature, dehydrated in ethanol, and embedded in paraffin wax. Tissue blocks were sectioned and stained by hematoxylin and eosin (H&E) for studying histopathological changes. Stained sections were photographed under light microscope at 400×.

Statistical analysis

Data were expressed as mean ± standard deviation (SD), and ANOVA test was used to analyze the difference among various treatments, with least significant difference at 0.05 and 0.01 as a level of significance using SPSS ver. 20.

Results

Body and testes weights

The initial body weight, final body weight, and testes weights are shown in Fig. 1. CCl_4 significantly decreased the final body and testicular weights compared with the control group ($P < 0.001$). The co-administration of CCl_4 + VE ameliorated the CCl_4 toxicity and protected

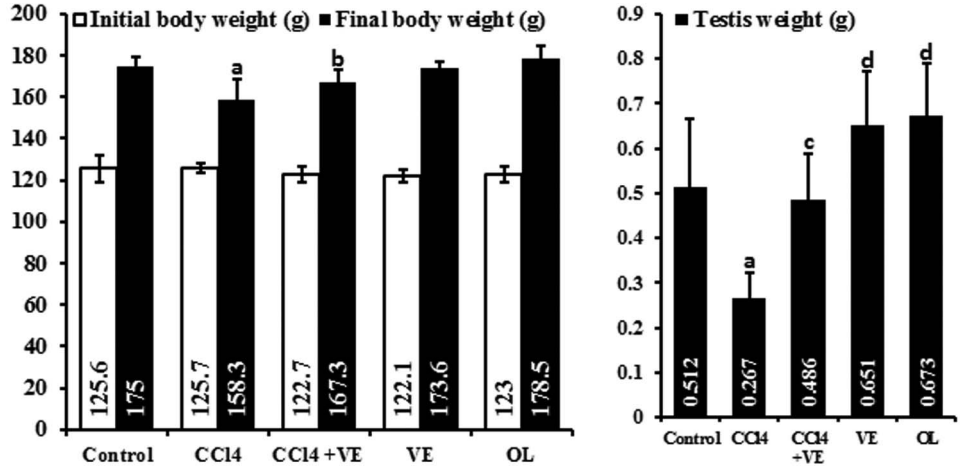

Fig. 1. Effect of VE on body and testes weights of CCl_4-treated rats. Data are means ± SD, $N = 10$. "a" denotes the significant differences ($P < 0.001$) vs. control group. "b" denotes the significant differences ($P < 0.01$) vs. CCl_4 group. "c" denotes the significant differences ($P < 0.001$) vs. CCl_4 group. "d" denotes the significant differences ($P < 0.05$) vs. control group. VE: Vitamin E-treated group and OL: olive oil-treated group

from adverse changes in the weights. However, a significant increase in testis weights was observed in VE and OL groups when compared with the control group.

Lipid peroxidation and anti-oxidative enzymes

The effects of VE against CCl_4-induced anti-oxidant status alterations are shown in Fig. 2. The testicular content of MDA significantly increased ($P < 0.001$), whereas SOD and CAT enzymes were significantly decreased ($P < 0.001$) after CCl_4 treatment compared with their respective control. The co-administration of $CCl_4 + VE$ ameliorated CCl_4 toxicity and significantly decreased ($P < 0.001$) MDA content, while it significantly ($P < 0.001$) increased SOD and CAT contents. However, a significant decrease in MDA and significant increase in SOD and CAT enzymes were observed in OL group when compared with their respective controls.

Serum levels of testosterone, LH, and FSH

Serum levels of testosterone and LH were significantly decreased ($P < 0.001$ and $P < 0.01$, respectively) in CCl_4-treated rats when compared with controls (Fig. 3), while the decrease in serum FSH was insignificant ($P > 0.05$). The co-administration of $CCl_4 + VE$ ameliorated the CCl_4 toxicity; sex hormone serum levels were improved but did not reach the mean control level. Significant increase ($P < 0.001$) was found in serum level of testosterone, while the increase in serum levels of LH and FSH was insignificant ($P > 0.05$) when compared with the CCl_4-treated group. VE and OL groups exhibited a significant increase in serum levels of LH when compared with the control group.

Sperm parameters

The sperm count, motility, and viability percentage were significantly reduced ($P < 0.001$) after treatment with CCl_4 compared with their respective controls (Fig. 4). However, the

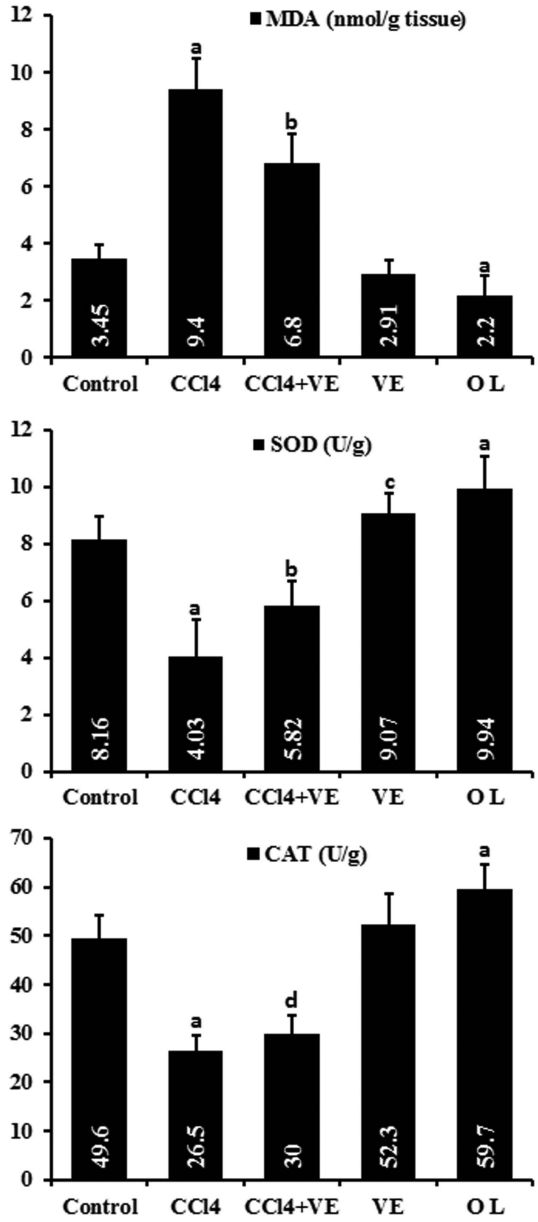

Fig. 2. Effect of VE on lipid peroxidation and anti-oxidant enzymes in testes of CCl_4-treated rats. Data are means ± SD, $N = 10$. "a" and "b" denote the significant differences ($P < 0.001$) vs. control and CCl_4 groups, respectively. "d" denotes the significant differences ($P < 0.05$) vs. CCl_4 group. VE: Vitamin E-treated group and OL: olive oil-treated group

co-administration of CCl_4 + VE significantly increased ($P < 0.001$) the spermatic parameters as compared with the CCl_4 group.

Histopathological findings

VE prevented the hazardous effects of CCl_4 on seminiferous tubules of rats, as shown in Fig. 5. The administration of CCl_4 for 4 weeks resulted in marked degeneration of spermatogenic layers and interlobular hemorrhage (Fig. 5b) when compared with the control

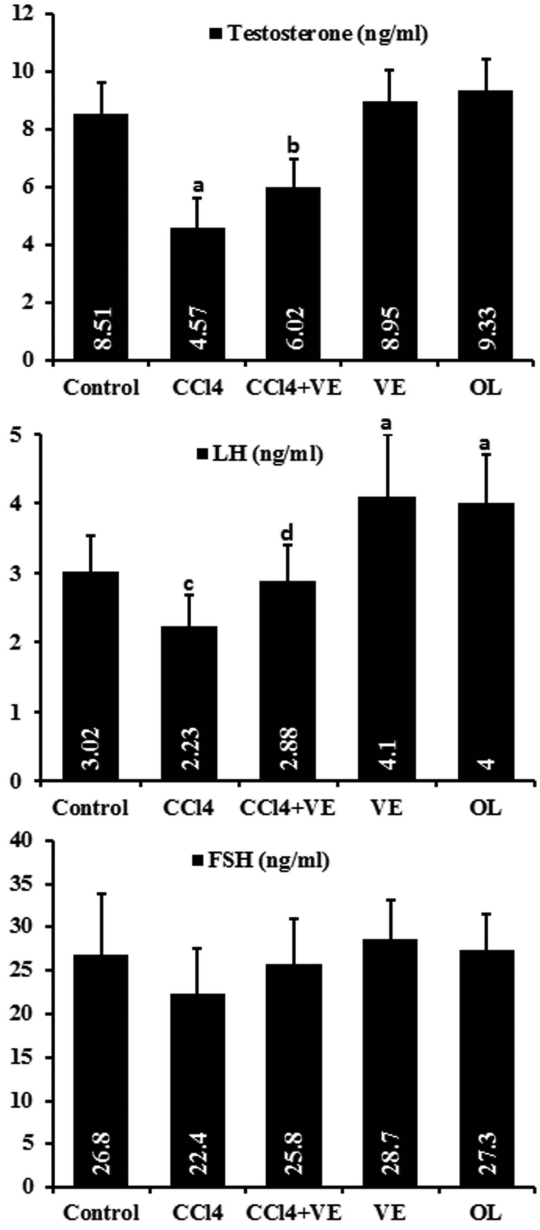

Fig. 3. Effect of VE on serum levels of hormones in CCl_4-treated rats. Data are means ± SD, $N = 10$. "a" and "b" denote the significant differences ($P < 0.001$) vs. control and CCl_4 groups, respectively. "c" denotes the significant differences ($P < 0.01$) vs. control. "d" denotes the significant differences ($P < 0.05$) vs. CCl_4 group. VE: Vitamin E-treated group and OL: olive oil-treated group

testis with normal seminiferous tubules and different types of spermatogenic cells (Fig. 5a). The co-administration of CCl_4 + VE (Fig. 5c) showed a significant repairing of testicular damage induced by CCl_4 with relative increase in the amount of sperms in the lumina, in addition to the regular arrangement of spermatogenic layers with fewer degenerations and more spermatogenesis compared with the CCl_4 group. The treatment with either VE or OL alone revealed a well-observed normal structure of seminiferous tubules and a regular arrangement of spermatogenic layers (Fig. 5d and e).

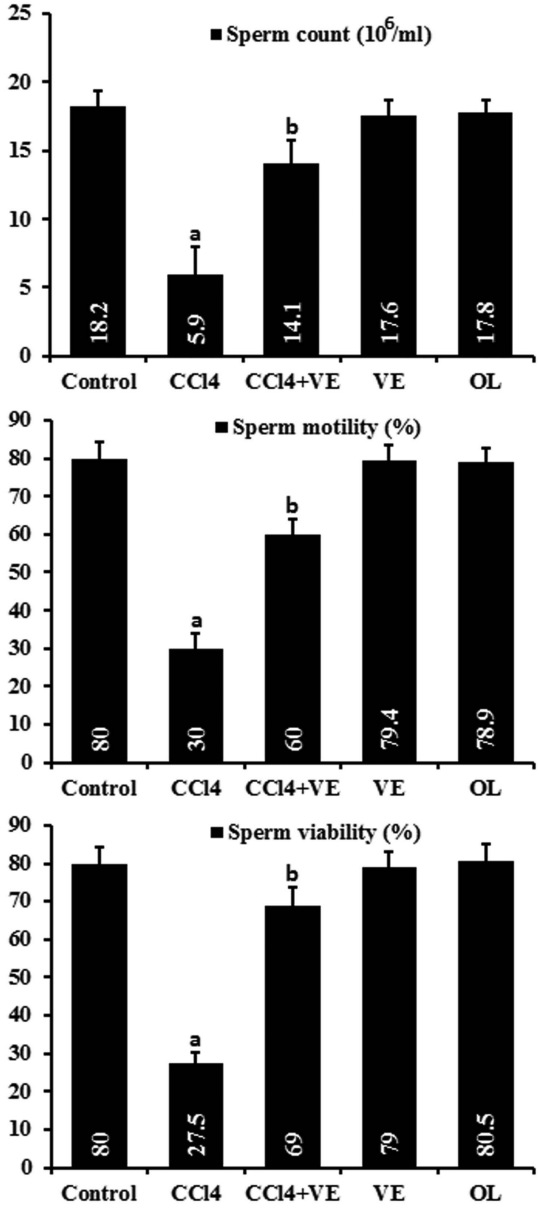

Fig. 4. Sperm characteristics of male rats in control and experimental groups. Data are means ± SD, $N = 10$. "a" and "b" denote the significant differences ($P < 0.001$) vs. control and CCl_4 groups, respectively. VE: Vitamin E-treated group and OL: olive oil-treated group

Discussion

VE is one of the primary components of the complex network of the anti-oxidant defense system of the spermatozoa. It is also one of the major membrane protectants that functions as a chain-breaking anti-oxidant preventing the propagation of lipid peroxidation (31). The dietary intake of VE can protect DNA, proteins, and lipids from oxidative damage (10). In male animals, testicular mass is an important parameter of reproductive toxicity and the decrease in testicular mass is always accompanied with the elimination of germinal cells (20).

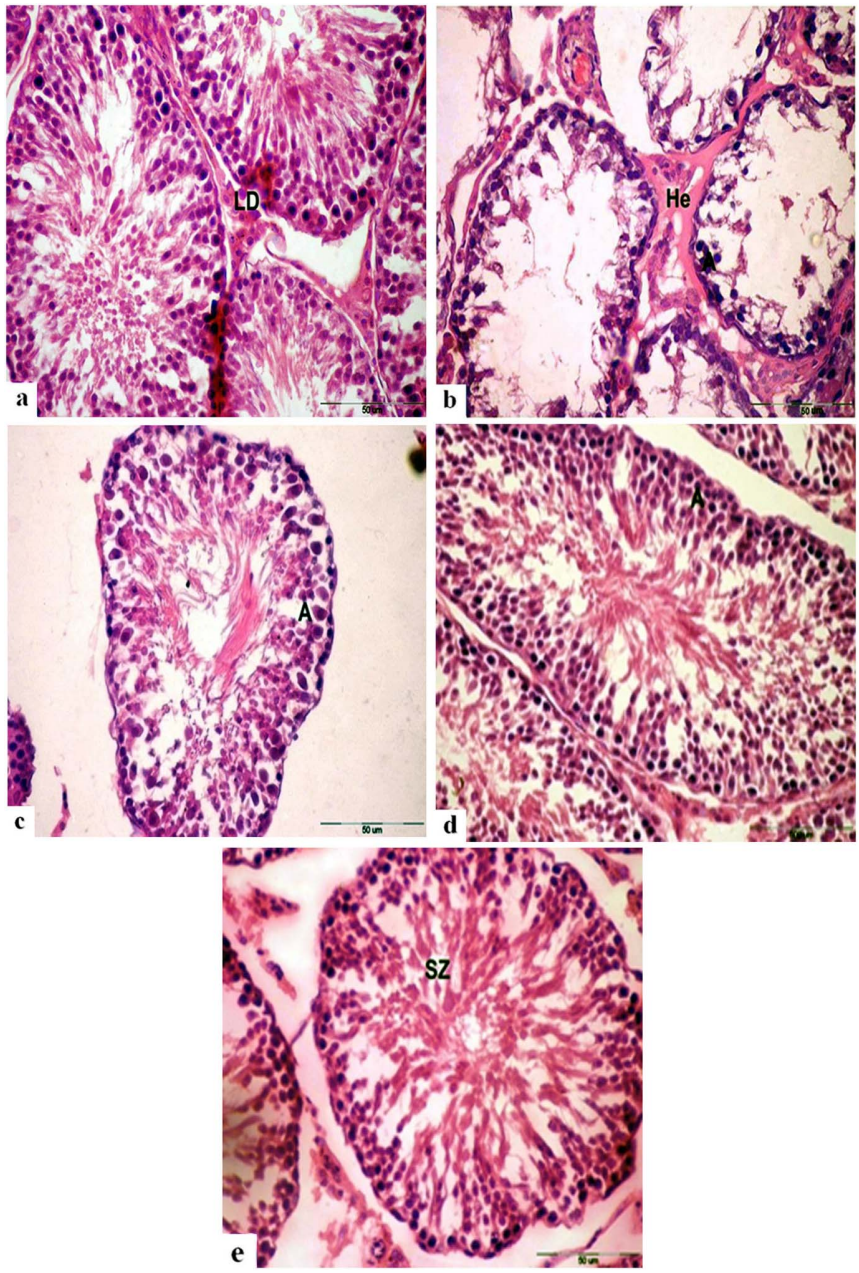

Fig. 5. VE prevented the hazardous effects of CCl_4 on seminiferous tubules of rats. Sections of testes of rats showing seminiferous tubules and different types of spermatogenic cells. (a) Control group showing normal seminiferous tubules, LD: Leydig cells. (b) CCl_4-treated group showing a marked degeneration of the spermatogenic layers and interlobular hemorrhage (He). (c) CCl_4 + VE-treated group showing a less degeneration of the spermatogenic layers (A) and more spermatogenesis compared with the CCl_4 group. (d) VE-treated group showing a regular arrangement of spermatogenic layers and a normal structure of seminiferous tubules. (e) OL-treated group showing a normal amount of spermatozoa (SZ) in the lumina, in addition to the regularly arranged spermatogenic layers (H&E 400×)

Sex hormones are among the reported biological markers known to evaluate the reproductive functions and toxicities in both animal and human subjects (34). In the present study, the administration of CCl_4 to adult male rats induced testicular toxicity manifested by alterations in reproductive functions evident by inhibiting spermatogenesis, testicular androgenesis, and gonadotropic secretion, as reported previously (21). CCl_4-treated rats exhibited a significant decrease in body and testes weights, as reported previously (19). However, others reported a significant increase in testis weight after CCl_4 administration which may be attributed to the different treatment times and dosage (20). There is a direct association between the epididymal sperm count, extent of spermatogenesis, and fertility in animals (2, 14). Sperm parameters such as count, motility, and viability are the key indices of male fertility, as these are the prime markers in testicular spermatogenesis, epididymal maturation, and sperm fertilization capacity (30). The significant decrease in sperm count, motility, and viability observed in the current study, as a result of CCl_4 treatment, is in agreement with the findings of others (19, 20).

Serum levels of testosterone, LH, and FSH were significantly lower in the CCl_4-treated group than those in the control group. CCl_4 may exert its effects either directly on Leydig cells or indirectly by altering the gonadal response to FSH and LH (23). In Leydig cells, LH stimulates the production of testosterone that activates the binding of FSH to Sertoli cells to stimulate spermatogenesis (7). It was reported that CCl_4-treated rats exhibited testicular dysfunction due to the fail of the pituitary gland to secrete FSH and LH (19). Reduction of LH, FSH, and intra-testicular testosterone levels can inhibit spermatogenesis leading to testicular dysfunction, as reported previously (40). Injuries of Leydig cells, as a result of testicular oxidative stress, can alter testosterone levels and is a common feature in infertility (9). One possibility for the adverse effect of CCl_4 on sperm parameters may be due to the decrease in the level of FSH, LH, or testosterone (29). Serum levels of testosterone, LH, FSH, body weight, testis weight, and sperm parameters were restored to near control level by VE supplementation to CCl_4-intoxicated rats. VE supplementation caused a significant increase in sperm count, viability, and protected the spermatozoa from the loss of motility. CCl_4 may affect the hypothalamic suprachiasmatic nucleus (SCN) that regulates the secretion of pituitary hormones. The increase in testosterone, FSH, and LH levels in the present study concurrent with the administration of VE + CCl_4 may be attributed to a direct effect of VE on the central nervous system, gonadal tissues, and/or hypothalamus–pituitary–testis axis (20). These results were in agreement with other studies having reported that VE stimulated LH and FSH release (17, 24). In addition, VE antagonized the decline of LH and FSH, testosterone, and sperm number; and reduced testicular injury as a result of dioxin treatment (41).

Here, CCl_4-treated rats exhibited a significant increase in testicular MDA, as have been reported previously (8, 15). The higher membrane lipid content of Leydig cell mitochondria and microsomes may explain the susceptibility of the testis to lipid peroxidation in CCl_4 exposed rats. In addition, mammalian spermatozoa are vulnerable to ROS damage due to their high membrane content of PUFA (11). The spermatozoa have an elaborate anti-oxidant defense system that scavenge and suppress the formation of ROS (6, 19). In the present study, activities of CAT and SOD in the testes were observed to be significantly reduced in CCl_4-treated rats. It has been reported that CAT is found in the peroxisomes of Leydig cells. Leydig cell peroxisomes participate in the intracellular cholesterol trafficking and delivery into mitochondria during LH-stimulated steroidogenesis in adult rat (26). Hence, the reduced CAT activity observed in CCl_4-treated rats may alter the process of steroidogenesis.

In addition, testicular injury may result from the interference of free radicals, generated as a result of CCl_4 treatment, with the anti-oxidant defense system (21). Furthermore, the increased level of lipid peroxidation is a consequence of depletion of anti-oxidant enzymes to certain critical levels, thereby shifting the delicate balance in favor of ROS leading to a plethora of pathologic damage to sperm cells and concomitant loss of function (37). The co-administration of VE with CCl_4 clearly restored the reproductive organ indices toward normal. VE ameliorated the decrease in the activities and levels of anti-oxidant enzymes (CAT and SOD), and the increase in lipid peroxidation (MDA) in testis of CCl_4-treated rats. This finding is consistent with the previous findings regarding the beneficial effects of VE against oxidative damage and biochemical alterations (12, 14, 42). In this scenario, free radicals are scavenged by VE to preserve the membrane fluidity and ion transport functions of the plasma membrane (5, 28).

In the present work, the histopathological results revealed marked alteration and degeneration of germ cells after CCl_4 treatment which may be a reason for the decreased testicular weights observed in the present and earlier reports (4, 15, 20). The action of CCl_4 on the testes may be ascribed as a direct toxic action of CCl_4 on the tissues and is likely to impair gonadal response to FSH and LH; and diminish the production of testosterone. VE supplementation resulted in remarkable restoration of the structural alterations in the testes as a result of CCl_4 treatment. This amelioration could be attributed to the capacity of VE to scavenge ROS and reduce the oxidative stress, as reported in the previous studies (41, 42).

Conclusion

Our results show that the protective effect of VE may be due to both an increase in the activity of the anti-oxidant defense system and an inhibition of lipid peroxidation and provides evidence that it may have a therapeutic role in free radical-mediated diseases. In addition, VE is not only able to compensate the adverse effects of CCl_4 on testes of rats, but it also increases sperm viability in such animals. Thus, on the basis of the above findings, it can be concluded that the treatment with VE ameliorates the severe physiological and pathological alterations in the testes of rats due to CCl_4 exposure.

Conflict of interest

The authors declare that there are no conflict of interest or financial disclosures.

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