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Research Article

Omega-3 Oil Ameliorate Histological and Ultrastructural Alterations Induced by Cadmium Chloride in Rats Testis

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ABSTRACT

Cadmium (Cd) is considered to be one of the major environmental pollutants which have potential threat to human health. Reports of declining male fertility have renewed interest in the role of environmental and occupational exposures in the etiology of human infertility. Cd exposure led to obvious degenerative changes in testicular tissue. This study was performed to investigate the Cd-induced structural effects on the testes and to evaluate the possible protective effect of omega-3 oil in adult albino rats. Thirty adult male rats were used in the present work, divided randomly into five groups, six rats in each group; the first group was considered as a control group and left without treatment except the standard rat chow and tap water. The second group was given 40 mg/L of $CdCl_2$ in drinking water while the third group was given 60 mg/L of $CdCl_2$ in drinking water. The fourth group was given 40 mg/L of $CdCl_2$ in drinking water plus omega-3 oil (4 g/kg diet), while the fifth group was given 60 mg/L of $CdCl_2$ in drinking water plus omega-3 oil (4 g/kg diet), the Cd-treated rats showed dose-dependent histological and ultrastructural alterations which have been ameliorated after exposure to omega-3 oil. The present investigation concluded that omega-3 played a protective role against Cd-induced histopathological changes in rat testis.

Keywords: Cadmium chloride, omega-3, testis, ultrastructural

INTRODUCTION

Cadmium (Cd) is a widespread toxic environmental and industrial pollutant. It is listed by the U.S. Environmental Protection Agency as one of 126 priority pollutants.[1] A great attention has been paid to Cd pollution by environmentalists due to its toxicity to plants, animals, humans, and even microorganisms.[2,3] Cd concentration in the environment has increased as a consequence of anthropogenic activity.[4] It is released into the environment by mining and smelting activities, atmospheric deposition from metallurgical industries, incineration of plastics and batteries, land application of sewage sludge, and burning of fossil fuels.[5] It concentrates along with the food chain and once absorbed irreversibly accumulates in the human body, particularly in kidneys, bones, respiratory tract, and other vital organs such as the lungs and the liver.[6]

Cd found to induce testotoxicity[7,8] through the production of reactive oxygen species and inhibition of antioxidant enzymes.[9,10] The protective role of omega-3 oil against the toxicity of several toxicants, some of them were minerals such as arsenic,[11] has been studied. However, as far searched, no such studies on the protective effect of omega-3 oil against Cd toxicity were found.

Cd is considered as one of the most toxic transition metal elements that can cause severe damage to testis,[7,8] induced severe necrosis followed chronic degeneration in rat testes, and also induced sex accessory gland atrophy and reduced circulating testosterone through reducing Leydig cell function.[12,13] However, its effectiveness dependent on the dose of exposed Cd.[14] Alhazza[15] showed that administration of 2.5 mg/kg S.C. $CdCl_2$ to male rats caused changes in testosterone and gonadotropic hormones. Cd accumulated in testes in response to time and dose increased oxidative stress enzymes, endocrine disruption, and altered apoptosis.[16] Cd given parenterally to pregnant laboratory animals induced a variety of adverse reproductive change, decreased litter size, resorption, fetal death, growth retardation, and congenital malformations in offsprings of exposed animals.[17] In female rats subcutaneously injection on the 6th day of gestation with different doses of $CdCl_2$ caused an increase in testes diameters

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of seminiferous tubules, a progressive sloughing of germ cells and vacuolization of Sertoli cells and Leydig cells hyperplasia in the pups were noted.[18]

Ercolani *et al*.[19] demonstrated a statistically significant decreased spermatogenesis and testosterone levels as well as discrete changes in Ca^{+2} homeostasis gene regulation in the presence of increasing levels of Cd in a rat model. Acute Cd exposure resulted in hemorrhagic injury to the testis, although some strains of animals were resistant to this effect.[20] Cd severely damaged the seminiferous tubules and caused degeneration and disintegration of spermatogenic cells. Leydig cells were also lost after Cd treatment.[21]

The testes of male albino rats intoxicated with $CdCl_2$ showed edematous swelling, congestion, hemorrhage, focal to multifocal white pale depressed areas of ischemic necrotic patches, increased oxidative stress, and histopathological and biochemical effects.[22,23] Several studies focusing on Cd-related changes in testicular histopathology have implicated testicular blood vessel damage followed by the degeneration of spermatopoietic epithelial, as the main cause of Cd toxicity.[24-26] Fouad *et al*.[27] found testicular damage induced by a single intraperitoneally injection of $CdCl_2$ (2 mg/kg), the mechanism by which Cd affected testis was oxidative stress and inflammation. Intraperitoneal injections of $CdCl_2$ caused a marked and prolonged reduction of spermatogenesis, accompanied by increased permeability of the blood–testis barrier.[28]

Cd has been found to cause obvious adverse effects on the proliferation of piglet Sertoli cells and caused their DNA damage, cell apoptosis, and aberrant morphology.[29] Treated rats with $CdCl_2$ at a single dose of 2 mg/kg B.W showed various degree of testicular degenerations such as degeneration and necrosis of germinal cells and irregularity and atrophy of seminiferous tubules.[30] Krichah *et al*.[31] and Toman *et al*.[32] showed that exposure to Cd also induced a pronounced alteration of spermatogenic process with dramatically reductions of spermatozoa produced in the lumen of the seminiferous tubules sections and a decrease of the intratubular tissue volume, vacuolation in the epithelium, and necrosis were appeared. De Souza Predes *et al*.[33] found that Cd significantly reduced the seminiferous tubules diameter and decreased volume density of seminiferous tubules. The tubules lumen was filled with degenerated germ cells and multinucleated spermatid aggregates, vacuolization of the seminiferous epithelium was also observed. By scanning electron microscopy (SEM) examination, the absence of spermatozoa and height diminishing of seminiferous epithelium were appeared.

A serious decrease in the level of testosterone, a significant elevation in serotonin, increased oxidative stress in testicular tissue, poor semen quality (sperm count, sperm motility, and sperm viability), and histopathological alterations in the testis of Cd-treated rats have been declared by several studies.[34-36] Cd exposure (1.1 μg/g of diet) to the male rats caused increased plasma testosterone levels significantly, while epididymal sperm concentration significantly decreased in contaminated rats as compared to correspondent controls.[37,38]

Zararsiz *et al*.[39] concluded that omega-3 FAs had favorable effects in rat testis tissue by preventing oxidative damage and increasing the level of testosterone. Omega-3 FAs have effective protection for testicular tissues against methotrexate-induced testicular toxicity male albino mice.[40]

Oral administration of omega-3 (2 ml/kg B.W) either before or after treatment with azathioprine was effective in decreasing the DNA fragmentation and total sperm abnormalities and significantly increasing the sperm count.[41] The data of Uygur *et al*.[42] suggested that fish omega-3 FAs pre-treatment may be beneficial for spermatogenesis following acute doxorubicin (DOX)-induced testicular damage by decreasing germ cell apoptosis and oxidative stress.

MATERIALS AND METHODS

Thirty adult albino male rats (*Rattus norvegicus*) (200–250 g and 8 weeks old) were used in the present study. They were bred and housed in plastic cages (56 cm × 39 cm ×19 cm), bedded with wooden chips in the animal house of Biology Department/College of Science/University of Salahaddin, Erbil. The animals were kept under standard laboratory conditions 12 h light:12 h dark with controlled temperature of 22 ± 2°C. The rats were given standard laboratory chow containing 0.5% NaCl, 22% protein, and 4–6% dietary fat[43] and drinking water *ad libitum*.

Experimental Design

The rats were divided randomly into five groups (each contained six rats), the first group serves as a control was the rats given standard rat chow and tap water, the second group was treated group given (40 mg/L $CdCl_2$ in drinking water), the third group given $CdCl_2$ (60 mg/L in drinking water), the rats in the fourth group were received $CdCl_2$ (40 mg/L in drinking water) + omega-3 oil (4 g/kg diet), the omega-3 oil purchased from local pharmacy, and the last group received $CdCl_2$ (60 mg/L in drinking water) + omega-3 oil (4 g/kg diet), the experiment period for all groups was 30 days.

For studying the effect of $CdCl_2$ and/or omega-3 oil, all animals were anesthetized with ketamine hydrochloride (100 mg/kg B.W.) and sacrificed at the end of each experiment. Testes were taken from the rats and were cut into small pieces (<0.5 cm^3 in thickness) and kept in the fixative.

Histological Preparations

Light microscopy (paraffin method)

Samples of the testes were directly fixed in Bouin's fluid for 24 h and then processed for paraffin method by dehydrating through ascending concentrations of ethanol (50%, 70%, 95%, and 100%), cleared in xylene, infiltrated in paraffin wax, and finally embedded in paraffin wax. Sections were cut at 4 μm thickness with a rotary microtome (Hunting Don, Bright. UK). The sections were stained by hematoxylin and eosin method.[44,45]

Electron microscopy

Tissue samples (1 mm^3) were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2–7.4 for 24 h, post-fixed in 1% osmium tetroxide for 1 h, dehydrated through a graded series of acetone (50%, 70%, 95%, and 100%), cleared in propylene oxide for 15 min (twice), infiltrated with propylene oxide plus

Araldite mixture (1:1) for 12 h, and then embedded in resin medium (Araldite CY 212: 10 g; Hardener 10 g; Accelerator DMP 30: 0.5 g; and di-n-butylphthalate plasticizer: 0.6 g). All these chemicals were obtained from Fluka and Bucha. Polymerization was accomplished in an oven at 60°C for 48 h.[46]

The blocks were cut by ultramicrotome (Reichert-Jung) into 0.5–1 μ thickness and stained by 1% toluidine blue in 1% borax for light microscopy. For transmission electron microscopy (TEM), 600–900A° sections were obtained and stained by 3% uranyl acetate and lead citrate.[47] These ultrathin sections were examined by (SHARP) TEM in Gazi University, Faculty of Medicine, Ankara, Turkey and (LEICA/Em FC6, CM12 Philips) TEM in UKM University, Science and Technology Faculty, Malaysia.

For SEM, testes were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2–7.4 for 24 h. After washing by the same buffer, they post-fixed in 1% osmium tetroxide for 1 h, dehydrated in ethanol (50%, 70%, 85%, 100%, and 100%). The samples were put in desiccator for air drying. After mounting, they were coated with gold by coating system (E5200 AUTO SPUTTER COATER),[48] then examined by (ZEISS, super A, 557P) SEM in UKM University, Science and Technology Faculty, Malaysia.

RESULTS

Both doses of $CdCl_2$ (40 and 60 mg/L) caused many histological and ultrastructural alterations in the testis, on the other hand, omega-3 oil was very useful in minimizing most of the $CdCl_2$ toxic effects.

As shown in Figure 1, a healthy histological structure of rat testis having a germinal epithelium undergoing cell division and well-formed spermatids is seen. Administration of low dose of $CdCl_2$ to the male rats has caused a histological damage to the germinal cells and also affected the spermatids production [Figure 2a and b]. The affected germinal cells appeared highly vacuolated and enlarged spermatogonia and showed Cd particles deposition [Figure 2c and d]. Further, damages due to $CdCl_2$ effect on the other cells of the interstitial space such as Leydig cells were noticed. Such effect included condensed and crescent-shaped nuclei which looked like characteristic apoptotic feature in addition to the appearance of a large number of vacuoles [Figure 2e and f]. These changes have been confirmed by the electron micrographs [Figure 3a and b], in which ultrastructural alterations were clearly appeared such as highly spermatids degeneration with shrunken nuclei, high number of vacuolation in all germinal

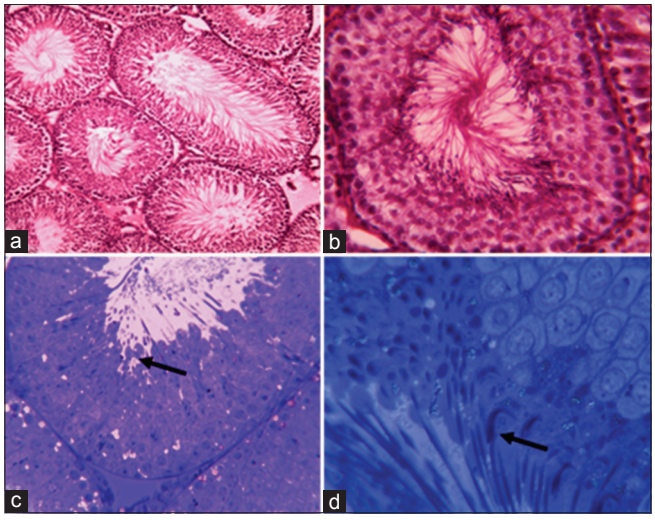

Figure 1. Sections through the testes of control rats, A&B) Paraffin sections showing normal histological structure of seminiferous tubules with a lot of spermatids in the lumen of the tubules, 100x and 400x respectively, H&E, C&D) Plastic sections showing normal germinal layer cells and the newly formed spermatids (arrow), 400x and 1000x respectively, toluidine blue.

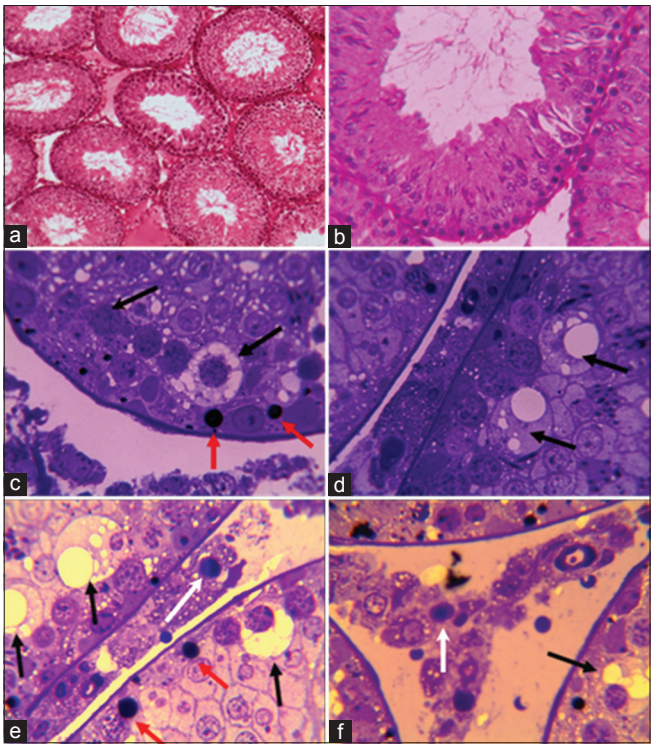

Figure 2. Sections through the testes of the low dose of CdCl2 treated rats, A and B) Low power of seminiferous tubules showing slightly empty lumen of seminiferous tubules 100x and 400x respectively, H&E, C-F) Different vacuolated spermatocytes (black arrows), degenerated Leydig cells (white arrow), precipitated Cd particles (red arrows) 1000x, toluidine blue.

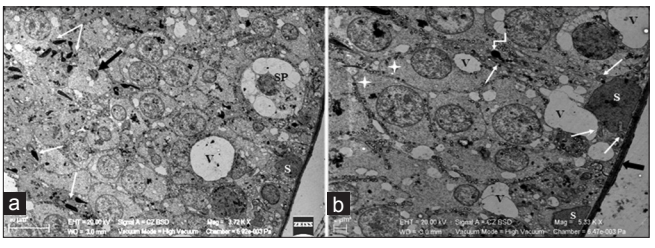

Figure 3. Electron micrographs through the testes of the low dose CdCl2 treated rats showing, A) Spermatid degeneration (white arrow), shrinkage of spermatocyte nucleus (black arrow), highly vacuolated spermatogonia (SP), vacuole (V), Sertoli cells (S). B) Thickining of basal lamina (black arrow), large and ✧small vacuoles in the germinal cells (V), shrunken Sertoli cells (S) and Cd particles (), notice the Golgi complexes (), different shape and size of mitochondria (white arrow), cup shape of spermatids head()

epithelium, especially the spermatogonia, thickening of the basal lamina, variation in the shape and size of mitochondria, and deposition of Cd particles.

Higher magnification of the later electron micrographs showed very large vacuoles with high deposition of Cd particles, lysis of mitochondrial membrane, high electron-dense sperm, and dilation of endoplasmic reticulum [Figure 4a and b]. Similar to the low dose, the high dose of CdCl2 caused similar damages to the germinal layer, in which no spermatids were seen in the lumen of the seminiferous tubules [Figure 5]. More alteration details were observed by electron microscopic images in the high-dose $CdCl_2$-treated rats which included approximately less spermatids production with defect heads and tails [Figure 6a], vacuolated and degenerated Leydig cells [Figure 6b].

A clear protective role of omega-3 oil was detected in the testis of $CdCl_2$-treated rats in both doses, in which an approximately normal histological structure of the seminiferous tubules was seen [Figure 7].

The scanning electron microscopic images have confirmed the histological and ultrastructural results regarding the testicular effect of $CdCl_2$ and the protective role of omega-3 oil [Figures 8 and 9]. As revealed by these figures, the cells of the germinal layer of the control group appeared filled the lumen of the seminiferous tubules with a large number of well-formed spermatozoa [Figure 8]. The little numbers of sperms and the presence of vacuolation in the germinal layer were clearly appeared in the testis of $CdCl_2$-treated group [Figure 9a].

The protective role of omega-3 oil against the $CdCl_2$ toxicity in the testis of male rat was clearly seen by the scanning electron microscopic image, in which a lot of spermatozoa were reappeared in the lumen of the seminiferous tubules [Figure 9b].

DISCUSSION

Spermatogenesis is a complex multitemporal process, including proliferation and differentiation of spermatogonia, meiosis, and spermiogenesis. In this process, any of the

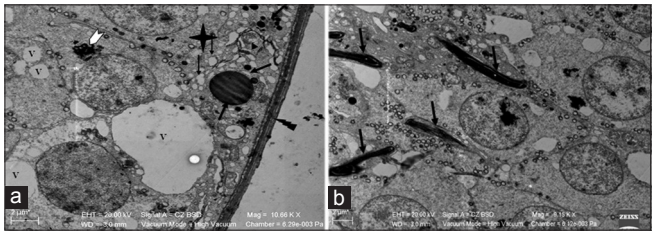

Figure 4: Electron micrographs through the testes of the low dose of $CdCl_2$-treated rats, (a) high deposition of Cd particles (thick arrow), thickening of basal lamina (⊲), lysis in mitochondrial membrane (head of arrow), spermatocyte with high electron density (➘), vacuolation in primary spermatocytes (V), and dilated smooth endoplasmic reticulum (thin arrow). (b) Degeneration of spermatid heads (black arrows) with vacuolation and lysed mitochondria (white arrow)

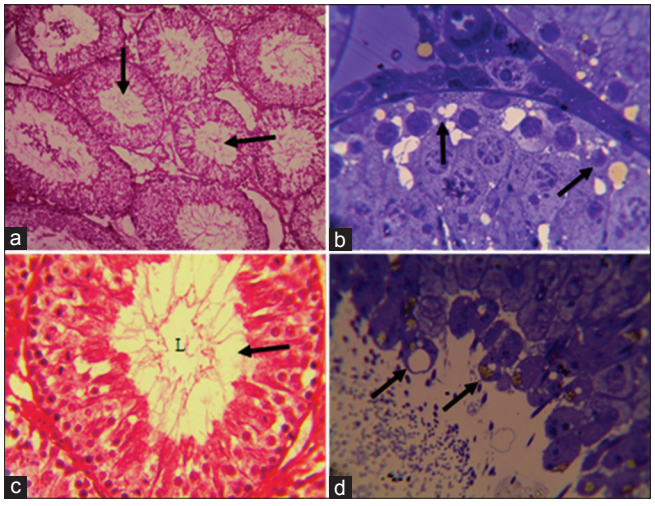

Figure 5: Sections through the testes of the high dose of $CdCl_2$-treated rats, (a) paraffin section through a number of seminiferous tubules showing empty lumen (arrows), ×100, (b) plastic section through a part of the germinal epithelium showing vacuolation of the cells (arrows), ×1000, (c) large lumen of seminiferous tubule and very little spermatids, ×400, (d) vacuolated spermatocytes with yellow lipid droplets (arrows) near the lumen (L), (arrows), ×1000

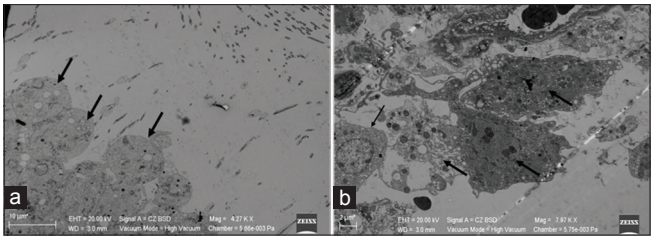

Figure 6: Electron micrographs through the testes of the high dose of $CdCl_2$-treated rats showing, (a) vacuolated spermatocytes (arrows), (b) healthy (thin arrow) and degenerated Leydig cells (thick arrow) showing a phagocytosis mechanism

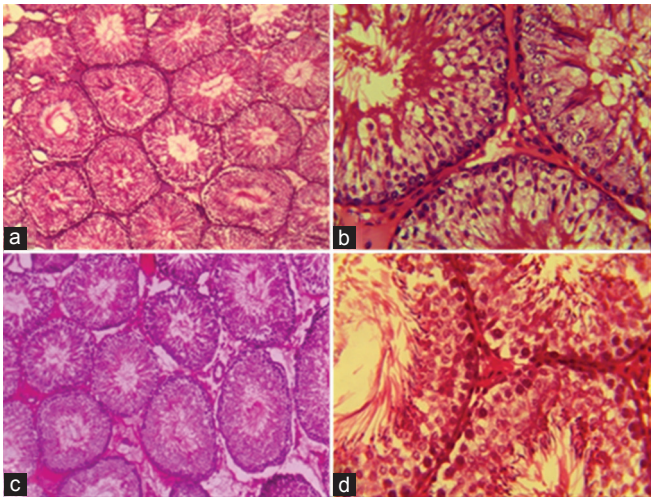

Figure 7: Paraffin sections through the testes of $CdCl_2$ plus omega-3 treated rats, (a) low dose of $CdCl_2$ plus omega-3 showing approximately normal structure of seminiferous with high quantity of spermatids in the lumen of the tubules, ×100, (b) higher magnification of the seminiferous tubules in the latter section, ×400, (c) high dose of $CdCl_2$ plus omega-3 treated rat group showing nearly normal seminiferous tubules, ×100, (d) same latter group showing number of spermatids and the healthy germinal cells undergoing cellular division, ×400, hematoxylin and eosin

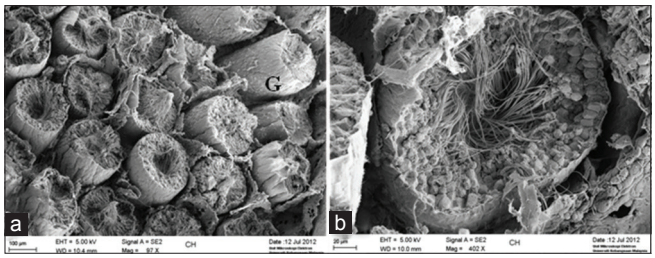

Figure 8: Scanning electron microscopy image through the testes of the rats in the control group showing normal seminiferous tubules containing high number of sperms (S), germinal epithelium (G), (a) low power, (b) high magnification

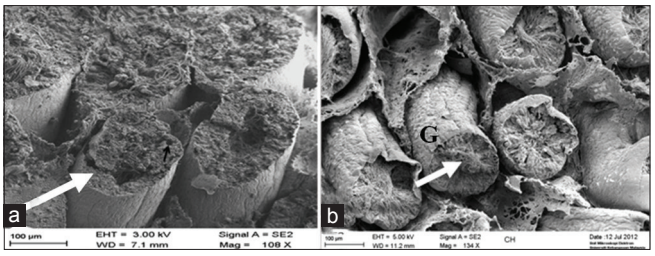

Figure 9: Scanning electron microscopy images of seminiferous tubules of (a) low dose of $CdCl_2$-treated rat testis showing little sperms in the lumen of the tubules and the formation of vacuoles in the germinal epithelial cells (arrows). (b) Low dose of $CdCl_2$ plus omega-3 treated group showing a lot of sperms in the lumen of seminiferous tubules (arrows)

affected areas are likely to cause spermatogenesis impairment and even infertility.[49,50]

Heavy metals could adversely affect the male reproductive system, either by causing hypothalamic-pituitary axis disruption or by directly affecting spermatogenesis, resulting in impairs semen quality.[51]

The present results showed different histological and ultrastructural alteration in rat testis, which included decrease in germinal epithelium thickness, slightly empty seminiferous tubule, and highly vacuolation features. These findings are in accordance with the previous observations.[30,52-53] Several studies focusing on Cd-related changes in testicular histopathology have implicated testicular blood vessel damage followed by the degeneration of spermatopoietic epithelial, as the main cause of Cd toxicity.[26,54]

Other studies on experimental animals have brought the evidence that oxidative stress is implicated in the toxicity of Cd.[55,56] The treatment with Cd (0.4 mg/kg B.W.) intraperitoneally caused significantly increased lipid peroxidation in rat tissues, enhancing peroxidation of membrane lipids altering the antioxidant system of the cells, which may cause injury to cellular components due to the interaction of metal ions with the cell organelles.[57]

Many ultrastructural defects on testes of rat testes were observed in the present study such as spermatid degeneration, shrinkage of spermatocytes, deposition of Cd particles, thickening of basal lamina, large and small vacuoles in the spermatocytes, and shrinkage of Sertoli cells. The later result concerning the effect on Sertoli cells was observed recently by Luca *et al*.[58] Kamel *et al*.[36] revealed that the testes are greatly targeted to damage by Cd intoxication and increased oxidative stress resulted from Cd intoxication in testicular tissue of treated rats might be responsible in testicular damage and impairment of fertility.

It has been reported that the oxidative stress affects the sperm cell through interfering with the membrane fluidity which is the main factor for sperm motility and fusion with the oocyte.[59] The present results showed decrease in the thickness of the germinal epithelium of the seminiferous tubules the testicular toxicity of Cd appeared to be mediated by a rapid apoptotic process as revealed by the decrease in the density of sperms in the lumen of the seminiferous tubules of treated rats. These observations agree with other studies indicating the implication of apoptosis in the mechanism of cytotoxicity of Cd in testes.[31,60] The data of Minutoli *et al*.[61] confirmed that the i.p. administration of $CdCl_2$-induced significant seminiferous epithelium damages and increased TUNEL-positive peripheral germ cells were observed in the seminiferous tubules, indicating an involvement of spermatogonia and primary spermatocytes in apoptotic processes. The previous studies of acute Cd exposure have reported diminished testicular weight in relation to the Cd dosage.[62] These studies attributed to this effect to the necrotic and degenerative changes induced by Cd.[63] The study of Toman *et al*.[30] demonstrated that Cd caused decrease in the thickening in the wall of the seminiferous tubules, and this may be due to the degeneration of germ cell layers.

Increase in the levels of total reactive oxygen species and lipid peroxidation was shown in testicular tissue after Cd administration, these biochemical alterations are considered as one of the forerunners of pathological changes in testis after Cd administration, also reduced level of enzymatic antioxidants and increased production of free radicals severely affected the testicular biology in the presence of Cd.[8] Testicular effects of Cd may be due to Cd interference with zinc-protein complexes that control DNA transcription, subsequently leading to apoptosis.[64] Most of the criteria of apoptotic mode of cell death in the germinal epithelium revealed in other studies that dealt with Cd testotoxicity (such as condensation of nuclear chromatin and degeneration of cytoplasmic organelles, especially the collapse of mitochondria)[50] were detected in the present work. Elmallah *et al*.[65] have been recently reported that oxidative stress and elevated levels of reactive oxygen species (ROS) are the main cause of cellular damage as a result of Cd exposure.

As revealed by the current investigation, omega-3 FO was succeeded in protecting the testis against most histological and ultrastructural changes caused by Cd. The most expected reason for this protection may be through oxidative/antioxidative mechanism. Similarly, Uygur *et al*.[42] suggested that fish omega-3 FAs pre-treatment may be beneficial for spermatogenesis following acute DOX-induced testicular damage by decreasing germ cell apoptosis and oxidative stress. Recently, Cd was found to decrease serum testosterone in rats.[7] Furthermore, omega-3 was found to elevate the level of testosterone in rats.[39] Such elevation of testosterone may the reason behind the protection of germ cells against the degenerative effect of Cd which has been detected in the present work since this hormone has been shown to regulate apoptosis in adult human somniferous tubules *in vitro*.[66]

CONCLUSION

The present investigation concluded that omega-3 oil could play a protective role against cadmium induced testicular toxicity due to the antioxidant power of the oil.

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