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Cadmium Toxicity in Spermatogenesis and Protective Effects of L-Carnitine in Adult Male Rats

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Abstract In this study, the effects of cadmium toxicity and the protective effects of L-carnitine on spermatogenesis in Sprague–Dawley rat were evaluated. Animals were subdivided into five groups. Cadmium chloride (1-mg/kg body weight) was injected intraperitoneally during 16 days at intervals of 48 h between subsequent treatments. L-Carnitine (500 mg/kg b.w., IP) was pretreated in both of control and cadmium-injected rats. Animals were killed on day 17 after the first treatment. The left cauda epididymis was removed and immediately immersed into Hank's balanced salt solution for evaluation of sperm count and viability. Following contamination with cadmium, a decrease in the number and viability of cauda epididymis sperm, the number of cell proliferation, and Johnsen Scores in the seminiferous tubules was observed. Consequently, L-carnitine treatment caused an increase in the number and viability of cauda epididymis sperm, the number of cell proliferation, and Johnsen Scores in the cadmium-induced group.

Keywords L-Carnitine · Cadmium toxicity · Spermatogenesis · Mature male rat

Introduction

Cadmium (Cd) is an important heavy metal widely used in batteries, metal plating, pigments, and the plastics in the alloy industries. In addition to occupational exposure, environmental Cd exposure in humans may occur through cigarette smoking and dietary consumption [1]. Acute Cd poisoning produces primarily hepatic and testicular injury, whereas chronic exposure results in renal damage and osteotoxicity [2].

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Severe testicular hemorrhage, edema, and necrosis with destruction of seminiferous tubules are the main testicular lesions due to Cd injection [3, 4]. Various mechanisms have been suggested to explain Cd-induced cellular toxicity. Carnitines have a protective role against reactive oxygen species (ROS) by exerting antioxidant properties [5]. Reactive oxygen species enhanced lipid peroxidation, altered antioxidant system, induced DNA damage, and altered gene expression and apoptosis [6].

It was reported that testis could be protected from toxic effects of Cd remarkably by mainly antioxidant treatment [7, 8]. L-Carnitine (LC) and acetyl-L-carnitine (ALC) are highly concentrated in the epididymis and play a crucial role in sperm metabolism, maturation, and the spermatogenic process [5]. Therefore, a combination of antioxidants appears to have a more profound effect against cadmium-induced testicular injury [9].

The objective of the present study was to examine the beneficial effects of LC on Cd-induced testicular damage. We evaluated the effect of Cd on spermatogonial cell proliferation in rat testes and the semen parameters (sperm concentration and viability) in cauda of epididymis.

Materials and Methods

Chemicals

Cadmium chloride was obtained from Sigma (St. Louis, MO, USA). L-Carnitine was obtained from Sigma (Tau, Pomezia, Rome, Italy).

Animals and Experimental Design

Thirty adult Sprague–Dawley rats (4–4.5 months old), weighing 200–250 g from Razy Research Center (Karaj- Iran), were used in this study. The animals were randomly divided into five groups:

- Group 1 (n=6): control animals.
- Group 2 (n=6): rats given distilled water (0.3 ml/body weight, intraperitoneal) for 16 days with an interval of 48 h between subsequent treatments.
- Group 3 (n=6): rats given L-carnitine (500 mg/kg b.w., IP) for 16 days with an interval of 48 h between subsequent treatments.
- Group 4 (n=6): rats given cadmium (1 mg/kg b.w., IP) for 16 days with an interval of 48 h between subsequent treatments.
- Group 5 (n=6): rats given LC + Cd in the same dose and time given to groups 3 and 4.

The animals were treated by LC 1 h prior to treatment with Cd. Cadmium administration dose was used according to a previous report [9]. Rats were housed under 12-h light, 12-h dark cycles. On the 17th day of the experiment, all animals were killed and samples were collected.

Handling of Testis Tissues

The pieces of left testes tissues were fixed in 10% formalin fixative and passed from increasing alcohol and embedded in paraffin. Sections of 4-μm thickness were stained by hematoxylin–eosin (H&E), and the germinal epithelium of at least 50 tubules was assessed according to a modified Johnsen Score under a light microscope (Table 1; Glander et al. [10]).

Table 1 Johnsen Score [10]

Score	Description
10	Full spermatogenesis
9	Many late spermatids, disorganized tubular epithelium
8	Few late spermatids
7	No late spermatids, many early spermatids
6	No late spermatids, few early spermatids, arrest of spermatogenesis at the spermatid stage, disturbance of spermatid differentiation
5	No spermatids, many spermatocytes
4	No spermatids, few spermatocytes, arrest of spermatogenesis at the primary spermatocyte stage
3	Spermatogonia only
2	No germ cells, Sertoli cells only
1	No seminiferous epithelial cells, tubular sclerosis

Immunohistochemical Study

All procedures for the immunohistochemical staining were performed by the Micro-Probe staining system, which is based on capillary action. Paraffin sections of 4 μm in thickness with a mounted probe on the slides were immunostained with antirabbit polyclonal antibody for the c-FLIP antigen (R&D systems, Inc., NJ, USA) by the avidin–biotin peroxidase complex method. The sections were deparaffinized and heated in a microwave oven for 7 min to retrieve the antigens. They were immersed in 0.6% hydrogen peroxide for 10 min to block the endogenous peroxidase activity. The primary antibody, at concentration of 1:100, was diluted in phosphate-buffered saline supplemented with 5% normal horse serum and 1% bovine serum albumin, and then this was incubated with the tissues overnight at room temperature. Antimouse immunoglobulin G (Sigma, St. Louis, MO, USA) labeled with biotin was used as a secondary antibody for the detection of the primary antibodies, and the slides were incubated for 10 min at 45°C with this secondary antibody. After multiple rinses with universal buffer, the streptavidin–alkaline phosphatase detection system (Biomeda, Foster, CA, USA) was applied for 8 min. As the final step, the slides were developed for 10 min with the enzyme substrate 3,3-diaminobenzidine (Sigma, St. Louis, MO, USA). The slides were counterstained with hematoxylin solution for 3 min (Research Genetics, Huntsville, AL, USA). After dehydration, the tissue was sealed with a universal mount (Research Genetics, Huntsville, AL, USA). For negative controls, the primary antibody was omitted and replaced with phosphate-buffered saline. Two hundred spermatogonial cells were counted in seminiferous tubules, and the percentage of spermatogonial cell proliferation activity was reported [11].

Sperm Parameters Assessment

The cauda of left epididymis was removed and immediately immersed in a petri dish containing 10 ml prewarmed Hank's balanced salt solution, and the sperms were allowed to diffuse into the buffer. After 5 min, the cauda of epididymis was removed, and the suspension was gently shaken to homogenize and spread the sperm [12, 13].

Sperm Count

A drop of resulting sperm suspension was used for the analysis of total sperm count. The sperm were counted using Neubaure chamber, as described by Belsy et al. [14].

Sperm Viability

Viability was assessed by eosin B (0.5% in saline). A 20-µl sample of the sperm suspension was placed on a glass slide, mixed with 7 µl eosin; coverslip was added and observed under a light microscope (×400 magnification). Because of the failure of the plasma membrane, the head of the spermatozoa absorb eosin and become red, but live spermatozoa remain colorless. Live spermatozoa were counted from each sample in five fields of vision randomly, and the percentage of live spermatozoa was recorded [12].

Statistical Analysis

The data were statistically analyzed by ANOVA and Tukey posttest. A P value of <0.05 was considered significant.

Results

Histological Findings

Histologic evaluation of testicular tissue was conducted in the sections dyed with hematoxylin–eosin and classified with Johnsen Score (Figs. 1 and 2). The Johnsen Score decreased significantly in groups 4 (Cd) and 5 (Cd + LC) in comparison with values of control group ($P<0.0001$ and $P<0.001$, respectively). In addition, the Johnsen Score increased significantly in the fifth (Cd + LC) group in comparison with the fourth (Cd) group ($P<0.0001$).

Immunohistochemical Findings

Proliferation activities were observed in spermatogonial series of seminiferous tubules with Ki-67. Proliferation activities were shown in spermatogonia of control and cadmium-treated rats (Fig. 3).The percentage of proliferating spermatogonial cells in seminiferous tubules decreased significantly in Cd-treated rats in comparison with that of control rats ($P<0.0001$; Fig. 4).

Sperm Parameters Findings

The average sperm number in cauda epididymal plasma decreased significantly in groups 4 (Cd) and 5 (Cd + LC) in comparison with values for control group ($P<0.0001$ and $P<0.001$, respectively). In addition, the average sperm number in cauda epididymal plasma increased significantly in group 5 (Cd + LC) in comparison with (Cd) group ($P<0.001$; Fig. 5).

A significant decrease in sperm viability was observed in Cd-treated rats, when compared with the control rats ($P<0.0001$; Fig. 6).

Discussion

Cadmium is a toxic metal which promotes oxidative stress and contributes to the development of serious degenerative changes in several tissues. It is well known that testes are very sensitive to acute Cd toxicity.

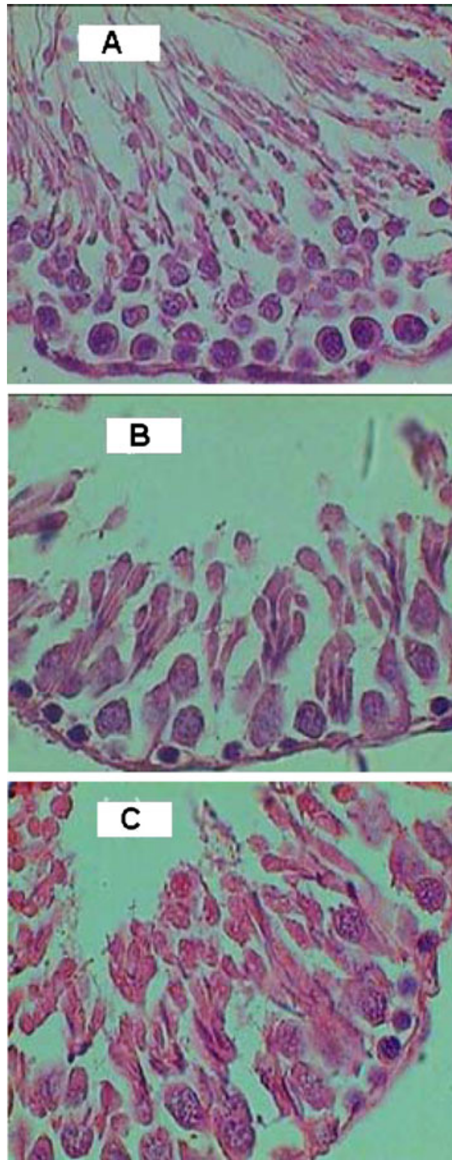

Fig. 1 The rat testes sections stained with H&E. **a** A testicular section from the control rat showing normal seminiferous tubules (foul spermatogenesis). **b** A testicular section from the rat treated with cadmium showing decreased spermatogenic cell population compared to control. **c** A testicular section from the rat treated with cadmium + L-carnitine showing increased spermatogenic cell population compared to cadmium-treated rats (fourth group). ×1,000

Various reports have shown that Cd induces oxidative stress by altering antioxidative status [6, 8, 15]. On the other hand, a study by Meral et al. [16] showed that a combination of antioxidants (vitamins C and E and selenium) has a large effect on damage caused by cadmium in testicular tissue. Similar studies also have reported on the treatment effect of antioxidants on testicular damages with Cd in animals [9, 17, 18]. As noted, LC and ALC

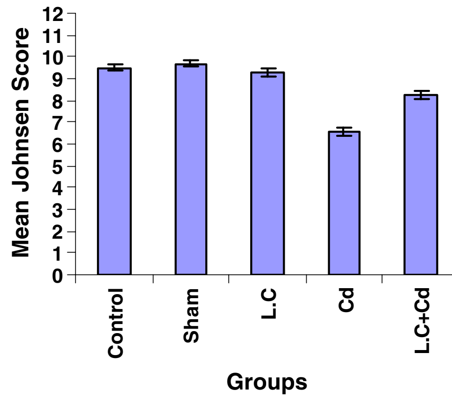

Fig. 2 The effect of L-carnitine on Johnsen Score in cadmium-treated male Sprague–Dawley rats (mean± SE, n=6). *P<0.0001, P<0.001 as compared to control group. **P<0.0001 as compared to cadmium (IV) group

are accumulated in the epididymis and have a main and determinant role on sperm metabolism, maturation, and the spermatogenic process and also have antioxidant properties [5].

Although a positive effect of some antioxidants on oxidative stress status due to Cd on testicular tissue was reported previously [16], the investigation of LC effect on cadmium

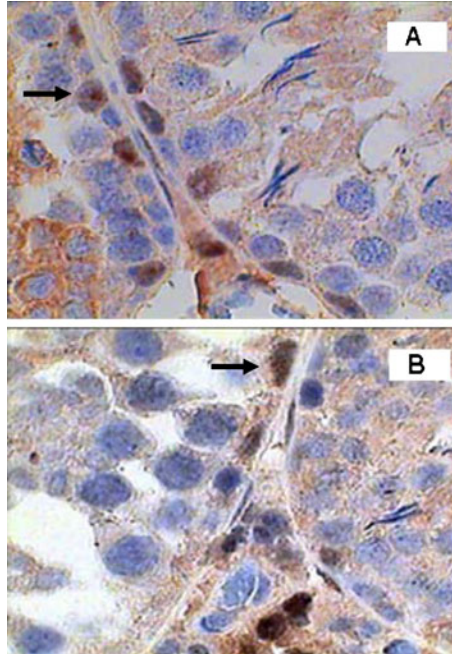

Fig. 3 Proliferation activity in testes as shown by Ki-67 staining in nuclei. Proliferation was seen in spermatogonial series of Cd (*arrow*; **a**) and Cd + LC groups (*arrow*; **b**). Concentration of the proliferation in Cd group is less than Cd + LC group. ×1,000

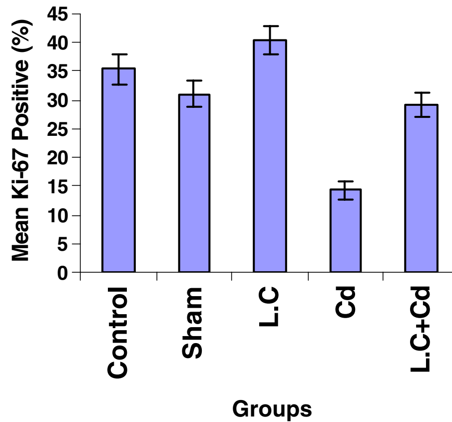

Fig. 4 The effect of L-carnitine on spermatogonial cell proliferation in cadmium-treated male Sprague–Dawley rats (mean±SE, $n=6$). *$P<0.0001$ as compared to control group

toxicity on testicular tissue has not been recorded. Thus, we decided to study the LC antioxidant effect on testicular tissue of rat treated with Cd.

Ki-67 antigen relates to nucleus protein and exposes in M steps at the end of G1, S, and G2 cellular cycle. Ki-67 antigen in mitosis is available on all chromosomes and emerges in perforated structure around metaphase chromosomes. This antigen is not distinguished in the beginning of the G1 stage and G0 stage and in DNA repair steps [19]. Therefore, its existence during cell cycle in the nucleus range indicates its importance in cell division. In the current research, based on the results in Cd-treated animals, spermatogonia cell proliferation had been reduced in seminiferous tubules. However, spermatogonia cell proliferation in seminiferous tubules showed an increase in the group treated with antioxidant (LC; Figs. 3 and 4).

Spermatogonium is a primary germ cell in seminiferous tubules, which is transformed to spermatozoa after proliferation [20]. Thus, continuing mitosis division in spermatogonia

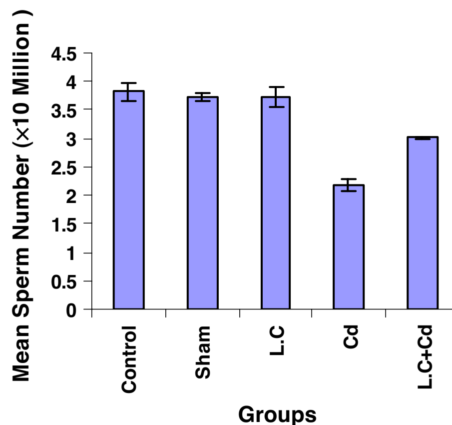

Fig. 5 The effect of L-carnitine on number of cauda epididymis sperm in cadmium-treated male Sprague–Dawley rats (mean±SE, $n=6$). *$P<0.0001$, $P<0.001$ as compared to control group. **$P<0.001$ as compared to cadmium (IV) group

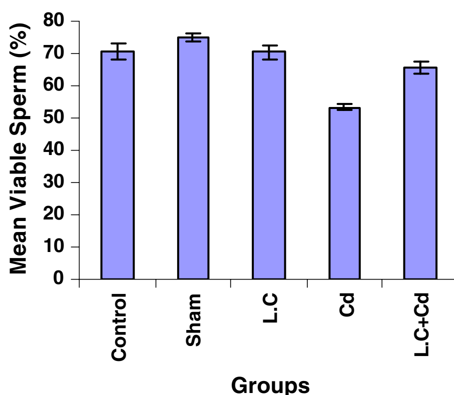

Fig. 6 The effect of L-carnitine on viability of cauda epididymis sperm in cadmium-treated male Sprague–Dawley rats (mean±SE, n=6). *P<0.001 as compared to control group

cells is important to support testicular tissue repair and number of sperm. Previous studies have shown that Cd may lead to a decrease in weight of reproductive organs, in the amount of testosterone hormone, and the number and motility of sperm in animals [21–23]. Akinloye et al. [24] investigated 60 infertile Nigerian men with azoospermia and oligospermia disorders and found that their seminal plasma Cd level had a significant increase level compared to control group.

In this study, it is shown that Cd has significantly reduced the number of epididymis sperm in the treated animals (Fig. 5). Reduction in the number of sperm in contaminated rats' epididymis is related to cellular population reduction present in seminiferous tubules and cell proliferation. Also, previous studies have shown that Cd leads to lower testosterone hormone production, which may be a secondary reason for reduction of cellular population in seminiferous tubules and number of sperms.

Cavallini et al. [25] found that prescribing edible LC and ALC is effective in idiopathic oligoastenoazospermia associated with varicose. Similar studies prescribing LC to infertile men showed that LC increased the average number and motility of sperms [17, 26, 27].

Our results showed that treatment with LC as an antioxidant against Cd toxicity increased the sperm number in comparison with cadmium-treated animals. It seems that LC as an antioxidant may prevent Cd damaging effects on a number of sperm probably by scavenging ROS.

Ramaĭia and Pomerantseva [28] in their study with mice reported that Cd causes cellular death for spermatocyte, spermatogonium, and hence infertility. Another study carried out by Kasinathan et al. [29] showed that Cd significantly reduces primary and secondary spermatocyte in seminiferous tubules.

Foote [30] reported that Cd reduces spermatogenesis and spermatogenetic elements necrosis in rabbit. Our results identified that Cd has reduced available spermatogenic cells population in seminiferous tubules of the treated animals (group 4; Figs. 1 and 2).

Cd exposure disturbs the cell cycle, cell proliferation, apoptosis, and DNA repair and reduces the expression of cell-proliferation-responsible genes [31]. Calmodulin is a regulatory protein that plays a major role in cell proliferation, and Cd interferes with this role [23]. Since available spermatogenic cells in seminiferous tubules have a good deal of cell proliferation, this process may be influenced by Cd, and cell proliferation may be inhibited and lead to spermatogenic cell population reduction in seminiferous tubules.

In one study, mice were first exposed to irradiation to deplete the spermatogonia and then were given LC. The sperm population in mice receiving LC showed faster improvement than controls. In early spermatogenesis stages, LC has a favorable effect on DNA repair and on proliferation of regenerated germ cells [5]. Therefore, in the group treated with Cd, LC may increase spermatogenic cell populations by increasing cell proliferation (Fig. 1 and 2).

Accordingly, average viable sperm percentage in cauda epididymis was reduced in animals receiving Cd (Fig. 6). Cell damage mechanism due to Cd exposures relates to DNA structural and functional change and membrane and oxidative damage [32, 33] because cell survival has a direct relation with DNA health and its membrane.

LC controls propagation of cell enzymes and consumption of oxygen in the cell [5]. Thus, it may prevent production of ROS and oxidative stress and increases cell viability.

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