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RESEARCH ARTICLE

# The Ameliorative Effects of L-Carnitine against Cisplatin-Induced Gonadal Toxicity in Rats

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**A B S T R A C T**

Cisplatin (CP) is a highly efficient remedy in cancer treatment, but it adversely affects the testicular tissue. This work assessed the ameliorative efficacy of L-carnitine (LC) against CP induced oxidative stress in rat testis, via investigating testosterone level and tissue oxidative/antioxidant parameters, histological alterations, and immunohistochemical expressions of intermediate filaments (IFs) proteins; vimentin (VIM) and cytokeratin 18 (CK18). Twenty-eight rats were assigned into four groups (7 rats each) as follows; groups I and II received saline and LC (100 mg/kg b.wt.) respectively orally once daily for 30 days; group III were injected with a single dose of CP (7.5 mg/kg, IP), 27 days after starting the experiment. Group IV received both LC and CP. Injection of CP significantly decreased serum testosterone and glutathione reductase and catalase in the testicular tissues and elevated malondialdehyde. Histologically, testes of the CP treated group revealed marked degenerative changes. Also, overexpression of both VIM and CK18 in testicular tissues were recorded. However, the administration of LC with CP restored the biochemical parameters, histological and immunohistochemical pictures towards the normalcy. Accordingly, LC is recommended as a supplement with chemotherapy to ameliorate its oxidative stress. This is the first study investigating the immunohistochemical expressions of IFs proteins, VIM and CK18, following administration of LC as a protective agent against CP induced testicular toxicity in rats.



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## INTRODUCTION

The testis plays a vital role in male reproductive function as it secretes testosterone, the male hormone, and responsible for androgenesis and spermatogenesis (Marty *et al*., 2003). Testosterone plays a critical role in growth, the appearance of sexual characteristics, maturation of male reproductive organs and spermatogenesis (Azarbarz *et al*., 2020).

Cisplatin (CP) is a potent anti-cancer medication used to treat a variety of tumors of the testes, ovary, bladder and lungs (Karwasra *et al*., 2016). However, several studies recorded that it induces testicular toxicity (Afsar *et al*., 2017; Almeer and Abdel Moneim, 2018; Azab *et al*., 2020; Azarbarz *et al*., 2020), inflammation, apoptosis, and oxidative stress (Meng *et al*., 2017) as well as, disorganization of the intermediate filaments (IFs) components of the cytoskeleton (Evans and Simpkins, 1998). Recently, vimentin (VIM) is considered as a mesenchymal marker for testicular toxicity and, cytokeratins (CKs) are known as cellular stress protein specially CK18 which is used as novel markers of testicular injuries (Banco *et al*., 2016).

Testicular dysfunction is the most reported consequence of CP toxicity, due to its high proliferative rate so the adverse effects of chemotherapy on the testis could be intense and irreversible causing the death of spermatogenic cells in the process of spermatogenesis and alterations in the sperm DNA, thus leading to the inability to generate a sufficient number of viable sperms (oligozoospermia), azoospermia or even prolonged sterility (Ekinci Akdemir *et al*., 2019; Azarbarz *et al*., 2020).

L-carnitine (LC) is a natural nutrient that is synthesized from lysine and methionine essential amino acids. It is derived from dietary sources (75%) and endogenous biosynthesis (25%). It presents in the epididymis in high levels and plays a vital function in spermatogenesis, spermatozoa maturation as well as metabolism (Abdel Aziz *et al*., 2018). LC is necessary for the production of ATP by β-oxidation of fatty acids in mitochondria (Aboubakr *et al*., 2020). Therefore, LC could prevent mitochondrial oxidative stress-induced by mitochondrial damage and apoptosis in different cell types (Barhwal *et al*., 2007). Accordingly, this work assesses the ameliorative efficacy of L-carnitine (LC) against CP induced oxidative stress in rat' testis via investigating testosterone and tissue oxidative/antioxidative parameters and revealing the histopathological alterations and immunohistochemical expressions of VIM and CK18 proteins.

## MATERIALS AND METHODS

**Chemicals:** Cisplatin was obtained from EIMC United Pharmaceuticals (Badr City, Egypt); each vial (50mg/ 50ml) was dissolved in physiological saline (0.9% sodium chloride). L-carnitine was obtained from MEPACO Company (Inshas Elraml, Egypt). Kits used for biochemical analysis (MDA, GSH, and CAT) were obtained from Biodiagnostics Company (Dokki, Giza, Egypt).

**Experimental animals:** The present study was carried out on 28 white albino male rats weighing 175-195 gm. Rats were obtained from the Center of Laboratory Animal at the Faculty of Veterinary Medicine, Benha University, Egypt. They adapted to the Laboratory of the Department of Pharmacology for two weeks before experimenting. Animals received a balanced commercial diet and water *ad libitum*. The study protocol was approved by the ethical committee of the Faculty of Veterinary Medicine, Benha University, Egypt.

**Experimental design:** Male albino rats were randomly separated into four equal groups/seven each, the control group, group I, received saline (the vehicle) orally, once daily for 30 days in a row. Group II, LC group, received LC (100 mg/kg b.wt.), orally once daily for 30 days in a row (Avsar *et al*., 2014). Group III, the CP group, was injected with a single dose of CP 7.5 mg/kg, via IP route on the $27^{th}$ day of the experiment (Boroja *et al*., 2018). Group IV, the LC+CP group, received a combination of treatments as both groups II and III**.**

**Sampling:** Twenty- four hours post-treatment; rats were anesthetized by inhalation of ether. Blood samples were collected by puncturing retro-orbital plexus in a sterilized dry centrifuge tube then left for 30 min at room temperature in a slanted position for coagulation before centrifugation at 1200 x g for 20 min to separate serum, which was stored at -20$^o$C until used for biochemical studies. Following blood collection, the animals of all groups were euthanized by cervical dislocation then both testicles were removed from each rat and thoroughly washed with physiological saline, then tissue homogenates were prepared (mentioned below) and centrifuged. The supernatants were isolated and used for evaluation of oxidative stress markers in testicular tissues; whereas the rest of the testicular tissues were preserved in neutral buffered formalin (10%) for histopathological and immunohistochemical investigations.

**Serum biochemical studies:** The serum testosterone level was quantified using an enzyme-linked immunosorbent assay (ELISA) kits (Immunometrics Ltd., London, UK).

**Preparation of testicular homogenates:** The tissue was dissected and washed with phosphate-buffered saline (PBS) solution, pH 7.4 containing 0.16 mg/ml heparin for removal of any and clotted red blood cells. Using a homogenizer, a gram of each testicular tissue was homogenized in 5 ml of 5-10 ml cold buffer, 50 mM potassium phosphate, pH7.5 1mM EDTA. Aliquots of tissue homogenates were centrifuged by cooling centrifuge 4000 rpm for 20min and stored at -20$^o$C till used for biochemical analysis.

**Detection of oxidative/antioxidant cascades:** Oxidative status was done by determination of the activity of glutathione reductase (GSH), catalase (CAT), and malondialdehyde (MDA) levels using special kits purchased from Bio diagnostic company, Egypt.

**Histological examination:** Testicular tissues were fixed in neutral buffered formalin (10%) for 48 hours. Then, specimens were dehydrated using ascending grades of alcohol, cleared in xylene, and embedded in molten paraffin. Five-micron thickness paraffin sections were cut and stained by hematoxylin and eosin for histological examination (Bancroft *et al*., 2013).

**Immunohistochemical studies:** A streptavidin-biotin complex (ABC) method was used to localize CK18 and VIM immunohistochemically. Antigen retrieval then blocking of nonspecific staining was carried out after dewaxing, rehydration, and blocking of endogenous peroxidase activity. The testicular sections were incubated with the primary antibodies, rabbit monoclonal anti-cytokeratin 18 and anti-vimentin antibody (Abcam, Boston, USA) at 1:200 dilution, for 1 hr at RT. Next, sections were incubated with biotinylated donkey anti-mouse IgG (Abcam, Boston, USA) for 30 min at RT. A commercial ABC system (Santa Cruz Biotech, CA, USA) was used for visualization of the reactions. The sections were then subjected to diaminobenzene (DAB) as the chromogen and counterstained with hematoxylin.

**Statistical analysis:** Statistical analysis was done using one-way ANOVA using the Duncan test, SPSS (Version 20.0; SPSS Inc., Chicago, IL, USA). The data were expressed as mean ± SEM and $P<0.05$ was considered significant.

## RESULTS

The biochemical parameters post-treatments were revealed (Table 1). Rats in the CP group had a significant decrease in the serum testosterone level when compared to those of the other groups. The data revealed a significant ($P<0.05$) increase in the MDA level along with a decrease in GSH and CAT in the testicular tissues of CP-intoxicated rats. Meanwhile, animals in the LC+CP group showed a significant ($P<0.05$) decrease in MDA level along with elevations in GSH and CAT in renal and hepatic tissues when compared to that of the CP treated group.

Histopathologically, both control and LC groups revealed normal histo-architecture of the seminiferous tubules and interstitial tissues. Normal arrangements of spermatogenic cells and Leydig cells were seen (Figs.1A, B). Meanwhile, CP treated group showed massive degeneration in some seminiferous tubules (Fig.1C), cytoplasmic vacuolization, reduction of germ cell layers, congestion of blood vessels in other tubules (Fig.1D), desquamation, and shedding of spermatogenic cells into tubular lumen (Figs. 1C, D) as well as widening of interstitial space with eosinophilic edema material (Fig. 1E). However, LC+CP treated group showed some improvements in the histological structure of both seminiferous tubules and interstitial tissues (Fig. 1F).

Immunohistochemically, most of the Leydig cells in both control and LC groups showed moderate CK18 immunolabeling (Fig. 2A, 2B). While very weak CK18 immunolabeling was seen in few Leydig cells of the CP treated group (Fig. 2C). An increase in the number and intensity of CK18 positive Leydig cells was identified in LC+CP treated group (Fig. 2D) when compared with that of the CP group. On other hand, strong VIM staining was observed in spermatogonia, spermatozoa, and Leydig cells in both control and LC treated groups (Figs. 3A, 3B), but CP treated group revealed a weaker response to VIM staining (Fig. 3C) compared to that control and LC groups. VIM staining nearly returned to normalcy in LC+CP treated group (Fig. 3D) compared with that of Fig. 3C.

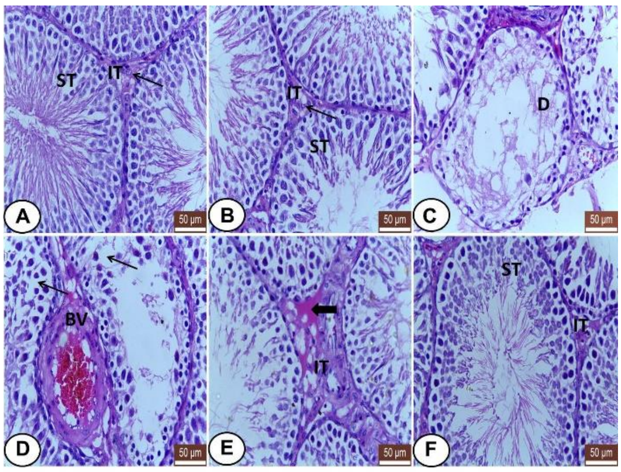


**Fig. 1:** Photomicrograph of testicular sections from all examined groups. A and B; Control and LC groups showed normal histo-architecture of the seminiferous tubules (ST) and interstitial tissues (IT). Notice, normal arrangements of spermatogenic cells, and Lydig cells (arrow). C-E; CP treated rats showed several histological changes. C; Massive degeneration in some seminiferous tubules (D). D; cytoplasmic vacuolization (arrows), reduction of germ cell layers, congestion of blood vessels (BV). Notice, desquamation, and shedding of spermatogenic cells into the tubular lumen of Figs. (C and D). E; widening of interstitial tissue (IT) with eosinophilic edema material (thick arrow). F; LC+CP treated rats showed some improvements in the histological structure of both seminiferous tubules (ST) and interstitial tissues (IT). H&E stain, scale bars=50μm.

## DISCUSSION

Most chemotherapeutics used for treating cancer induce toxicity and oxidative injury in different organs as testes (Azarbarz *et al*., 2020). In the present work, CP significantly lowered serum testosterone levels. Such a result could be explained as the Leydig cell dysfunction, which produces gonadotropin as well as decreasing the activity of both mitochondrial side-chain cleavage as well as cytochrome $P_{450}$ (García *et al*., 2012). Also, CP causes adverse effects on the function of Sertoli cells and lowers the androgen-binding protein expression. Furthermore, hormonal disorders caused by CP are mediated by its effects on the hypothalamic-pituitary-gonadal axis (Almeer and Abdel Moneim, 2018). A similar finding was recorded in previous studies (Afsar *et al*., 2017; Almeer and Abdel Moneim 2018; Azab *et al*., 2020). Moreover, CP-induced reduction in testosterone levels was significantly reverted by L-carnitine administration in the current work. The positive impact of L-carnitine on the level of L-carnitine

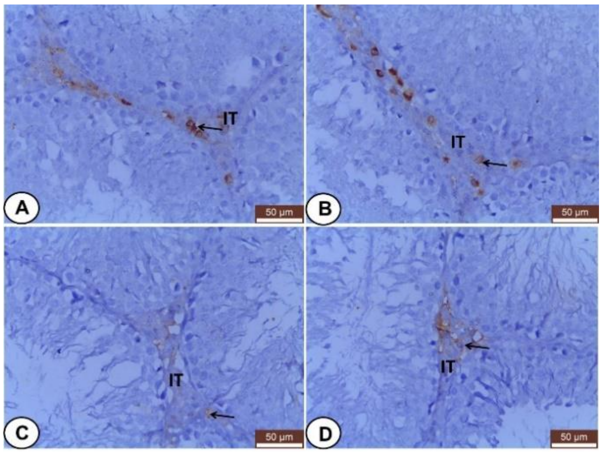


**Fig. 2:** Photomicrograph of CK18 immunostaining in testicular sections from all examined groups. A and B; Control and LC groups showed moderate CK18 staining. C; CP group revealed very weak and few positive CK18 cells. D; LC+CP group showed an increased number and intensity of CK18 positive interstitial cells. Leydig cells (thin arrow); interstitial tissue (IT). Scale bars = 50μm.

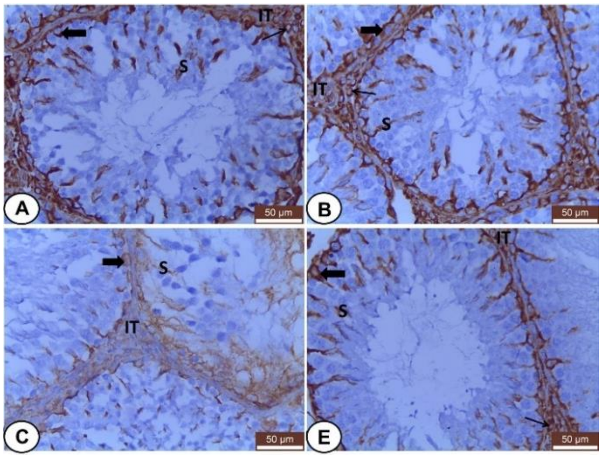


**Fig. 3.** Photomicrograph of VIM immunostaining in testicular sections from all examined groups. A and B; Control and LC groups showed strong VIM staining in spermatogonia, spermatozoa, and Leydig cells. C; CP group revealed weak and few positive VIM cells. D; LC+CP group showed the increased intensity of VIM positive cells. Leydig cells (thin arrow); interstitial tissue (IT). Spermatogonia (thick arrow), spermatozoa (S), and interstitial tissue (IT). Scale bars = 50μm.

**Table 1:** Effect of CP and/or LC treatment on serum testosterone and oxidative stress markers in testicular tissues in rats (n=7)

| Parameters | Control | LC | CP | LC+CP |
|---|---|---|---|---|
| Testosterone (ng/ml) | 2.38±0.09[b] | 2.29±0.03[b] | 1.23±0.06[a] | 2.04±0.19[b] |
| MDA (nmol/g) | 57.84±1.51[c] | 55.74±1.37[c] | 92.46±3.40[a] | 79.21±2.38[b] |
| GSH (mg/g) | 4.85±0.21[a] | 4.78±0.05[a] | 3.26±0.14[c] | 4.03±0.05[b] |
| CAT (U/g) | 29.26±0.79[a] | 28.88±0.46[a] | 17.80±0.52[c] | 25.74±1.42[b] |

LC, L-carnitine at the dose of 100 mg/Kg PO; CP, cisplatin at the dose of 7.5 mg/Kg IP; MDA, malondialdehyde; GSH, reduced glutathione; CAT, catalase. Data are expressed as the mean ± SE. Different superscript letters in the same row indicate statistical significance at P≤0.05.

on the level of testosterone may be explained as its anti-oxidative activity which counteracts the oxidative stress-induced Leydig cell damage (Ghanbarzadeh *et al*., 2014).

In this study, CP considerably elevated MDA and depleted GSH, CAT, and SOD activities in the testicular tissue. A similar imbalance was recorded (Anand *et al*., 2015) indicating that the levels of antioxidant enzymes were insufficient for eliminating free radicals produced by CP (Azarbarz *et al*., 2020). Such reduction of antioxidant enzymatic molecules might be because of an uncontrollable generation of $H_2O_2$, which impairs antioxidant defense systems of the testis. Results of the present work come along with those of previous investigations (Asfar *et al*., 2017; Ekinci Akdemir *et al*., 2019; Yadav, 2019). However, treatment with LC counteracted the oxidative stress of testes and enhanced the testicular antioxidant defense system, representing that LC suppresses oxidative stress in testes (Ghanbarzadeh *et al*., 2014). Also, LC reduces lipid availability for peroxidation by transporting fatty acids to the mitochondria for β-oxidation and consequently mitigates the production and accumulation of lipid peroxidation products (Aboubakr *et al*., 2020). LC is a natural antioxidant acting as a free radical scavenger (Abdel Aziz *et al*., 2018). Furthermore, LC could regulate carbohydrate metabolism and preserve the structure of the cell membrane, cellular vitality, and it is considered as an essential cofactor in the process of long-chain fatty acids oxidation (Caloglu *et al*., 2009).

Histologically, the testicular specimens of both control and LC groups showed normal histo-architecture for the seminiferous tubules and interstitial tissues. Similar findings were reported (Eid *et al*., 2016; Aktoz *et al*., 2017). CP administration causes massive degeneration, cytoplasmic vacuolization, and reduction of spermatogenic cell layers, congestion of blood vessels, desquamation, and shedding of spermatogenic cells into the tubular lumen as well as edema of interstitial space. such findings were reported (Almeer and Abdel Moneim, 2018; Gevrek and Erdemir, 2018; Prihatno *et al*., 2018). Such impairment of spermatogenesis might be due to a remarkable reduction of the testosterone level in addition to, the increased production of free radicals due to severe damages of Leydig cells (Tousson *et al*., 2014; Kaya *et al*., 2015). After administration of LC to CP treated group, structural improvement of the seminiferous tubules and interstitial tissue was observed indicating tissue repair which was similar to findings of Eid *et al*. (2016). These findings could be attributed to the anti-oxidative property of LC that prevents oxidative-stress induced Leydig cell impairment; consequently, LC can restore testosterone level (Ghanbarzadeh *et al*., 2014). Noteworthy, LC improves histopathological changes in the ipsilateral testis of albino rats (Gawish *et al*., 2011). Also, Ahmed *et al*. (2014) and Yuncu *et al*. (2015) reported that LC prevents spermatic changes after CP exposure.

Immunohistochemically, low expressions of both CK18 and VIM in the testicular tissue after CP administration were observed in the current study. A similar finding is observed by Prihatno *et al*. (2018). Additionally, this study reported the restoration of CK18 and VIM in the testicular tissue of the LC+CP group indicating the protective role of LC against CP-induced testicular histopathological changes.

**Conclusions:** The present study revealed the adverse effect of CP on rat testis as inflammation and structural alterations through induction of oxidative stress determined by increased generation of MDA and reduced activity of antioxidant enzymes. This is the first study, according to our knowledge, to investigate the immunohistochemical expressions of IFs proteins, VIM, and CK18, following administration of LC as a protective agent against CP induced testicular toxicity in rats. It is recommended to supplement LC to protect the testes against CP induced toxicity due to its antioxidant and anti-inflammatory properties.

**Authors contribution:** AS, HR, AE, and MA; contributed to the study design, experimental work, and statistical analysis and writing the manuscript. Mahmoud AE; performed the histopathological and immunohistochemical parts of the study. SF and EA; analyzed the sera and tissue samples. HK; critically revised the manuscript for important intellectual contents and submitted the manuscript. All authors interpreted the data and approved the final version.

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