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

Adham Sallam1, Hanan A. Rizk4, Mahmoud Abdelghaffar Emam2, Sabreen Ezzat Fadl3, Ehab Yahya Abdelhiee6, Hanem Khater7*, Ashraf Elkomy1 and Mohamed Aboubakr2

1Department of Pharmacology; Faculty of Veterinary Medicine, Benha University, 13736 Moshtohor, Toukh, Qaliobiya, Egypt; 2Department of Histology; Faculty of Veterinary Medicine, Benha University, 13736 Moshtohor, Toukh, Qaliobiya, Egypt; 3Department of Parasitology; Faculty of Veterinary Medicine, Benha University, 13736 Moshtohor, Toukh, Qaliobiya, Egypt; 4Department of Pharmacology, National Organization for Drug Control and Research (NODCAR), Egypt; 5Biochemistry Department, Faculty of Veterinary Medicine, Matrouh University, 51744 Matrouh, Egypt; 6Forensic Medicine and Toxicology Department, Faculty of Veterinary Medicine, Matrouh University, 51744 Matrouh, Egypt

*Corresponding author: hanemkhater@gmail.com; hanem.salem@fvtm.bu.edu.eg

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ABSTRACT
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.

RESEARCH ARTICLE

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 27th 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°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°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.

**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<sub>450</sub> (Garcia 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...
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$_2$O$_2$, 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 $\beta$-oxidation and consequently mitigates the production and accumulation of lipid peroxidation products (Aboubaker 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 testosteron 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 spermatogenic 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.

**REFERENCES**


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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>LC</th>
<th>CP</th>
<th>LC+CP</th>
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<tbody>
<tr>
<td>Testosterone (ng/ml)</td>
<td>2.38±0.09$^a$</td>
<td>2.29±0.03$^b$</td>
<td>1.23±0.06$^c$</td>
<td>2.04±0.19$^c$</td>
</tr>
<tr>
<td>MDA (nmol/g)</td>
<td>57.84±1.51$^b$</td>
<td>55.74±1.37$^b$</td>
<td>92.46±3.40$^a$</td>
<td>79.21±2.38$^b$</td>
</tr>
<tr>
<td>GSH (mg/g)</td>
<td>4.85±0.21$^a$</td>
<td>4.78±0.05$^b$</td>
<td>3.26±0.14$^c$</td>
<td>4.03±0.05$^c$</td>
</tr>
<tr>
<td>CAT (U/g)</td>
<td>29.26±0.79$^c$</td>
<td>28.88±0.46$^c$</td>
<td>17.80±0.52$^a$</td>
<td>25.74±1.42$^b$</td>
</tr>
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LC, L-carnitine at the dose of 100 mg/Kg IP; 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.


