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Clinical Research

Effects of L-Carnitine on iNOS Expression and Apoptosis in CCL4-induced Testicular Damage in Rats

Derya Karabulut[1*], Ayca Kara[2], Ali Tugrul Akin[3], Tugce Merve Ozturk[1], Murat Unsal[1], Emin Kaymak[4]

[1]*Erciyes University, Faculty of Medicine, Department of Histology-Embryology, Kayseri, Turkiye.*

[2]*Erciyes University, Genome and Stem Cell Center (GENKOK), Kayseri, Turkiye.*

[3]*Istinye University, Faculty of Medicine, Department of Medical Biology, Istanbul, Turkiye.*

[4]*Bozok University, Faculty of Medicine, Department of Histology-Embryology, Yozgat, Turkiye.*

Abstract

Purpose: *This study aimed to investigate the effects of L-carnitine on inducible nitric oxide synthase (iNOS) and apoptosis in testicular damage caused by carbon tetrachloride (CCL4).*
Methods*: Forty male Sprague Dawley rats were used. Experimental groups; Group I: Control group; Group II: L-carnitine; 200 mg/kg L-carnitine (ip) twice a week for 6 weeks, Group III: CCL4; 0.2 ml CCL4 (ip) twice a week for 6 weeks, Group IV: L-carnitine + CCL4 group, 200 mg/kg ip L-carnitine was administered 24 hours before CCL4 administration twice a week for 6 weeks, Group V: CCL4+L-carnitine; 200 mg/kg L-carnitine was administered half an hour after CCL4 administration twice a week for 6 weeks. Histopathology, Johnsen's testicular biopsy score (JTBS), seminiferous tubule diameter measurement, iNOS immunoreactivity and apoptosis, which is programmed cell death, were evaluated in testicular tissue sections.*
Results: *Compared to Group I, histological deterioration, decreased JTBS and MSTD, increased iNOS expression and apoptotic cells were observed in Group III tissue sections. All of these parameters showed corrective effects in L-carnitine applied groups.*
Conclusion: *L-carnitine has both protective and healing effects on tissue against CCL4 toxicity by helping regulate iNOS expression.*

Key words*: Carbontetrachloride, L-carnitine, iNOS, Testis*

*** Corresponding author:** Derya Karabulut, E-mail: deryakkus@hotmail.com, ORCID ID: 0000-0003-2067-6174

Introduction

Causes such as alcohol-cigarette use, drug consumption, aging, varicocele, urogenital infections, and exposure to toxic chemicals are risk factors for male infertility (1, 2).

In such cases, the severity of the dysfunction result from tissue or organs is affected by variety of factors depends on the duration that structure is affected by those factors. Carbon tetrachloride (CCl4) is a colorless, volatile, toxic substance that is rapidly miscible in air, water and soil (1). It is used as a solvent in industrial industry, but also in agriculture as a disinfectant for cereals and as a pesticide (3). Direct or accidental ingestion of CCl4 may cause serious damage in the testicular tissue using high accumulation ability (4). CCl4 is frequently used in experimental animal models at different doses especially to induce experimental liver fibrosis (5, 6). Moreover, it has been reported that CCl4 has detrimental effects on other organs such as the liver (7), heart (8) and testis (9). CCl4 causes the emergence of harmful metabolites such as trichloromethyl in the cell and directly affects the mitochondrial membrane by blocking the oxidative phosphorylation of free radicals containing an unpaired electron (10, 11). Nitric oxide (NO) is a free radical that can be covalently bound to other molecules because it has an unpaired electron in its final orbit (12). NO is synthesized from L-arginine by the activity of nitric oxide synthase enzymes (NOS) (13). There are three forms of NOS: neuronal NOS (nNOS, NOS-I), inducible (iNOS, NOS-II) and endothelial NOS (eNOS, NOS-III) (14, 15). The iNOS gene is a Ca 2+ and calmodulin-independent molecule encoded by 1153 amino acid proteins, consisting of 26 exons, 25 introns and 130 kDa in weight (13). It is expressed in testis, epididymis, endothelial cells, smooth muscle cells, hepatocytes, chondrocytes, keratinocytes (16), mast cells and monocytes, macrophages, microglia, Kupffer cells and also expressed in phagocytic cells such as eosinophils and neutrophils (17). Revealing whether iNOS, which is also expressed in the testis, changes against CCL4 toxicity is necessary to understand the presence of NO enzymes caused by oxidative stress in the tissue.

L-carnitine is a white, water-soluble substance with good thermostability, which can be found anywhere in nature, synthesized from plant and animal cells. L-carnitine fulfills its catalytic function by the formation of fatty acids in mitochondria, and its metabolic function as a buffer for excess acyl residues (16). L-carnitine protects the cell membrane and DNA against damage caused by free oxygen radicals, and prevents protein oxidation and

lactate oxidative damage (17). Therefore, it is important to reveal the presence of iNOS expression due to oxidative stress caused by CCL4 toxicity of L-carnitine, which is known for its protective effects on the cell. Because increased oxidative stress in the cell can cause disruption of physiological processes and even trigger cell death. In this regard, changes in apoptosis, which is programmed cell death due to iNOS expression, can also be observed in CCL4-induced testicular damage.

In this study, we aimed to evaluate the damage caused by CCL4 on the testis and the protective and ameliorative effects of L-carnitine against this damage by histopathological evaluation, immunohistochemical stainings and the determination of apoptosis.

Methods

Animals

The study used 40 male Sprague Dawley rats. The rats were obtained from Erciyes University Experimental and Clinical Research Center (DEKAM), Kayseri, Turkey. The rats were adult, 2-3 months/ 8-12 weeks old, weighing 200-300 g. This study was performed according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (date: 04.04.2024, number: 24/072). The cages were well-ventilated and plastic. The rats were allowed to have ad libitum access to food and water. They were also housed in a room with a 12-hour light:dark cycle. At the end of the experiment, they were anesthetized under ketamine + xylazine anesthesia and their organs were removed.

Experimental design

Group I: Control group (n:8) Olive oil was given intraperitoneally (ip) twice a week. Group II: L-carnitine group (n:8), 200 mg/kg L-carnitine (ip) was administered twice a week for six weeks (18). Group III: CCL4 group (n:8) was applied by dissolving twice a week in 0.2 ml of CCL4 (ip) olive oil for 6 weeks (19). Group IV: L-carnitine+CCL4 group (n:8) were given 200 mg/kg L-carnitine (ip) 24 hours before each CCL4 administration during the experiment. 24 hours after L-carnitine administration, 0.2 ml/100 g CCL4 was applied. Group V: CCL4 + L-carnitine group (n:8), 200 mg/kg L-carnitine (ip) was administered half an hour after CCL4 twice a week (20). Since CCL4 was dissolved in olive oil, it was deemed appropriate to give olive oil to the control group. At the end of the experiment, the testicular tissues of the rats were removed under ketamine (75 mg/kg) + xylazine (10 mg/kg) anesthesia.

Histological Examination

At the end of the experiment, the testicular tissues were placed in a 10% formaldehyde solution. Then, after waiting for a week in the fixation solution, they were dehydrated using alcohol. Transparent with xylol and embedded in paraffin. Hematoxylin-Eosin (H-E) staining and iNOS immunoreactivity were applied to 5 μm thick sections to evaluate the histological structure. Testicular tissue sections were subjected to Johnsen's testicular biopsy score (JTBS) by two independent histologists. According to JTBS, testis sections were systematically evaluated. This is done by giving a score of 1 to 10 for each section. Score descriptions are as follows; 1: There are no cells in the tubular section. 2: There are only Sertoli cells. 3: Germ cells are only spermatogonium. 4: There are few spermatids. 5: There is a large number of spermatocytes. 6: There are few spermatids. 7: There is a large number of spermatids without signs of discernment. 8: Late spermatids are present without mature spermatozoa. 9: There are few spermatozoa. 10: Complete spermatogenesis with a large number of spermatozoa (21). In addition, using the Analyses LS Research program at testicular tissue sections, the mean seminiferous tubule diameter measurement (MSTD) was performed at x20 magnification with 100 tubules from each group.

Hematoxylin-Eosin (H-E) Staining Protocol

Paraffin sections were incubated at 58 ºC. The sections were incubated in xylene and washed with alcohol. Sections washed in running water at room temperature for 10 minutes in hematoxylin solution was kept. Sections were washed again in running water. The Eosin solution were kept for 5 minutes. The washed sections were first dehydrated and then incubated in xylene (20).

Immunohistochemistry

Immunohistochemistry was performed with the avidin-biotin-peroxidase method to determine iNOS (ab3523, Abcam, Cambridge, UK) immunoreactivity in testicular tissue. 5 μm thick sections were taken from the paraffin sections. Then, these sections were kept in the oven to remove excess paraffin. Before staining, the sections were placed in an xylene solution and deparaffinized. The sections were rehydrated with alcohol and rinsed in deionized water. Then, antigen retrieval was carried out by microwave treatment in 0.01 M sodium citrate buffer (pH 6.0) at 95°C for 5 min. The tissue slides were washed with phosphate-buffered saline (PBS). Endogenous peroxidase activity was

inhibited by 3% H_2O_2 in methanol for 10 min. The staining kit (Lab Vision, Ultra Vision Detection System Large Volume, Anti-Polyvalent Thermo Scientific HRP) was used for the next stages according to the manufacturer's instructions. The sections were visualized using 3,3P-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin (20). Under the light microscope (Olympus BX51, Center Valley, PA, USA) and images were obtained. A total of 80 different fields (8 slides x 10 fields) in each group were evaluated using the ImageJ program.

Apoptosis (TUNEL)

Samples were stained by the directives of the manufacturer (In Situ Cell Detection Apoptosis Fluorescein Kit, Roche) to evaluate apoptosis. First, deparaffinization was performed with xylene from 5 mm sections. Then, dehydration was performed using alcohol. PBS was used for subsequent washing procedures. Testis slides antigen retrieval was applied for removed formaldehyde. After being washed with PBS three times, the tissues were incubated with a TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) reaction mixture in a damp and dark place at 37°C for 60 minutes. After washing with PBS three times for five minutes, the tissues were contrast colored with 4,6-diamidine-2'-fenilindol. They were all examined with the Olympus BX-51 fluorescent microscope. TUNEL-positive cells in seminiferous tubules of all groups were analyzed by counting (22).

Statistical analysis

For statistical analysis, GraphPad Software, Prism 10 version was used. D'Agostino & Pearson normality test was used to determine the normality analysis of the data. One-way ANOVA and post hoc Tukey testis were used to compare multiple variables. $p<0.05$ was considered significant. Data were evaluated as ± standard deviation.

Results

Histological results

Testicular tissue sections of groups I and II had normal histological appearance. Spermatogonium, Sertoli cells, primary and secondary spermatocytes, spermatids and spermatozoa had a regular appearance. Seminiferous tubules and inter-tubular connective tissue had a normal appearance. There was deterioration and vacuole formation in the seminiferous tubule germinal epithelium of group III. Also, in some tubules atrophy and necrotized germinal epithelium were observed. JTBS and seminiferous tubule diameter measurements were the lowest in this group. Tissue sections of Groups IV and V had a more regular appearance compared

with Group III. The tissue sections of Group V were more regular with seminiferous tubule germinal epithelium. The statistical analyses of the JTBS and MSTD scores of the experimental groups are shown in Table 1. The findings of the groups in the H-E stained sections are shown in Figure 1.

Table 1. Analysis of JTBS and MSTD scores of all groups.

	Group I	Group II	Group III	Group IV	Group V	*p*
JTBS (1 to 10)	8,94±0,87[a]	9,11±0,87[a]	7,15±1,05[b]	8,83±0,82[a]	8,83±0,58[a]	0,0001
MSTD (µm)	228,7±43,15[a]	223,9±32,77[a]	221,5±31,02[b]	231,2±29,18[a]	229,2±35,45[a]	0,0001

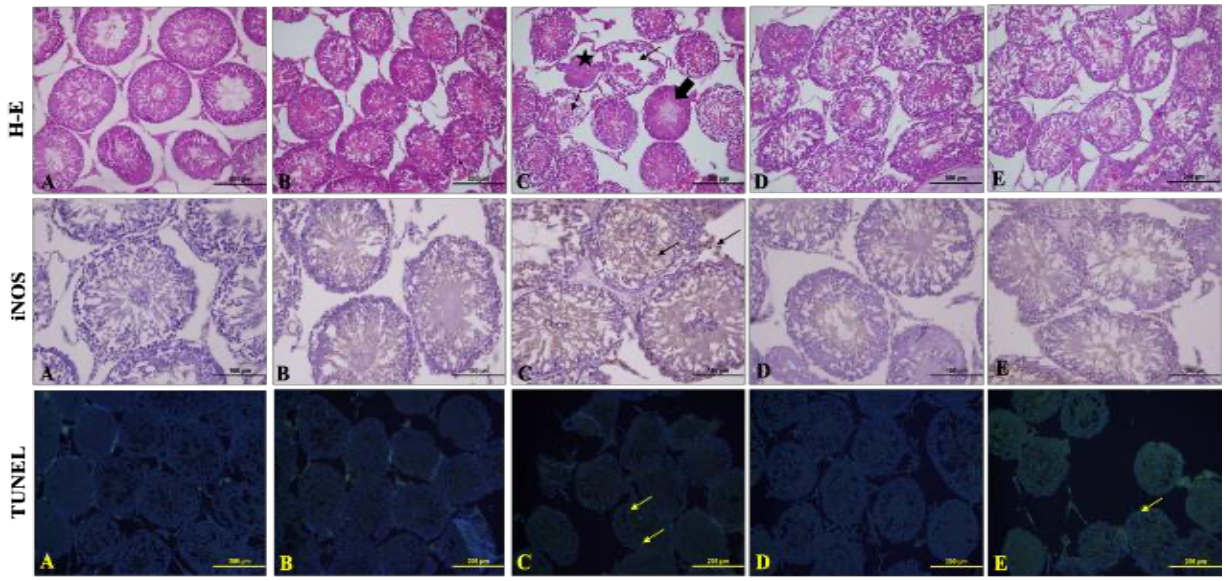

Figure 1. H-E, iNOS and TUNEL staining testicular sections of all experimental groups. Typical histological architecture of testis tissue in H-E stained sections Group I (A) and Group II (B). Atrophic seminiferous tubule (star), spilled germinal epithelium (arrow), and necrotic tubule (bold arrow) in Group III (C) were shown. Groups IV (D) and Group V (E) appear similar to normal testicular histology. Increased iNOS immunoreactivity (black arrows) is shown in Group III (C) compared to other groups. TUNEL-positive cells (yellow arrows) showing apoptosis are shown in Group III (C) and Group V (E). Abbreviations: Group I; Control group, Group II; L-carnitine group, Group III; CCL4 group, Group IV; L-carnitine + CCL4 group, Group V; CCL4 + L-carnitine group, iNOS; inducible nitric oxide synthase, TUNEL;Terminal deoxynucleotidyl transferase dUTP nick end labeling.

Immunohistochemistry results

iNOS immunoreactivity was observed in both the seminiferous tubule germinal epithelium and tubular connective tissue in the testicular tissue section. No statistically significant difference was observed in Group I and Group II. A statistically significant increase was found in Group III compared to Groups I and II. iNOS immunoreactivity was decreased in Group IV compared to Group III, but this decrease was still not statistically significant. Group IV and Group V iNOS immunoreactivities were statistically similar. No statistically significant difference was found between Group V and Group II. iNOS immunoreactivity results are shown in Table 2. Testicular sections stained with iNOS are shown in Figure 1.

Table 2. Analysis of iNOS immunoreactivity and TUNEL-positive cell count of all groups.

	Group I	Group II	Group III	Group IV	Group V	*p*
iNOS immunoreactivity	131,19±77,78[a]	132,46±94,00[ad]	140,97±82,87[b]	137,23±90,37[bc]	136,52±72,70[cd]	0,0001
TUNEL Positive cell count	0,08±0,34[a]	0,12±0,38[a]	1,82±1,42[b]	0,20±0,53[a]	0,16±0,46[a]	0,0001

Data are expressed as mean±standard deviation. There was no significant difference between groups containing the same letter (a and b). $p<0.05$ was considered significant. Abbreviations: Group I; Control group, Group II; L-carnitine group, Group III; CCL4 group, Group IV; L-carnitine + CCL4 group, Group V; CCL4 + L-carnitine group, iNOS; inducible nitric oxide synthase, TUNEL;Terminal deoxynucleotidyl transferase dUTP nick end labeling.

Apoptotic results

TUNEL-positive cells were observed in both seminiferous tubules and inter-tubular connective tissue. According to the cell count results, the TUNEL-positive cell number was increased in Group III when compared with other groups. There was a decrease in the number of TUNEL-positive cells in both L-carnitine groups and the values were close to Groups I and II. Statistical analyses of TUNEL-positive cell counts of the experimental groups are shown in Table 2. TUNEL-positive cells are shown in Figure 1 as FITC+DAPI (merge).

Discussion

CCl4 has been reported to inhibit spermatogenesis depending on the dose and duration of administration, altering

reproductive function by damaging testicular androgens and gonadotropic secretion (9). The underlying cause of testicular damage is the cytochrome p450 system, which causes the formation of trichloromethyl radicals. CCl4 metabolites react with polyunsaturated fatty acids to form covalent binds with cell membrane elements, resulting in testicular damage (23). In our study, tissue sections belonging to the CCl4 group were also damaged especially seminiferous tubules and spermatogenic series cell group. The JTBS and seminiferous tubule diameter measurements that we used for tissue evaluation were significantly decreased in Group III compared to Group I. Depending on the dose and duration of CCl4 administration, it is inevitable that the testis will not be damaged. In order to reduce the negative effects of various diseases and toxic agents on the reproductive system, various plant extracts, drugs, vitamins, etc. agents were used (2, 22, 24, 25). L-carnitine was found to be effective in both JTBS and MSTD in both the protective and therapeutic groups. L-carnitine plays an important role in lipid metabolism by facilitating long-chain fatty acids and is also known to protect the cell, mitochondrial membrane and DNA integrity against free oxygen radicals (26). We found significant improvements in tissue against CCl4 damage in L-carnitine-treated groups (Group IV and V). In our study, in addition to impaired testicular histomorphology in Group III, iNOS immunoreactivity also increased in both seminiferous tubules and inter-tubular connective tissue. iNOS is induced in a wide range of tissue and cell types to which cytokines and bacterial products are exposed. It is a factor in vasodilatation and inflammation. iNOS is stimulated by proinflammatory cytokines such as lipopolysaccharide and tumor necrosis factor-α and β, interleukin-1α. The role of iNOS in the immune system is very complex, it has both protective and toxic effects (27, 28). Our results show that CCl4 is applied regularly for 6 weeks causing testicular tissue damage and increased iNOS level mediates damage. Oxidative stress and free radicals in tissue cause molecules such as NO to increase (4). NO is continuously synthesized from L-arginine through NOS enzymes, resulting in increased NO accumulation due to increased levels of iNOS. The reduced iNOS immunoreactivity in L-carnitine-treated groups may be due to its function as an agent against oxidative stress in the tissue. L-carnitine protects DNA and membranes against free radicals with its antioxidative effect. Thus, it prevents the formation of reactive oxygen species and protects germ cells (29). Increased production of free radicals and decreased antioxidant defense system cause

accumulation of radicals in the cell and strengthen the formation of testicular apoptosis (26). In our study, an increase in the number of apoptotic cells in the testicular tissues of Group III was observed. The number of apoptotic cells decreased in L-carnitine-treated, Group IV and Group V. L-carnitine has been shown to have an anti-apoptotic effect in diseases such as ischemia-reperfusion, diabetes, and toxic conditions such as atrazine-exposure (26, 30, 31).

Conclusions

In our study, we showed that CCL4 causes testicular damage according to JTBS, MSTD, iNOS immunoreactivity and apoptosis findings. As a result, it is demonstrated that L-carnitine (200 mg/kg ip) may have a strong protective and therapeutic effect against CCL4-induced testicular damage via iNOS.

Conflict of interest

No potential conflict of interest was reported by the author(s)

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