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HISTOLOGICAL AND IMMUNOHISTOCHEMICAL EVALUATION OF PROPHYLACTIC VERSUS THERAPEUTIC EFFECT OF L-CARNITINE ON SUBMANDIBULAR SALIVARY GLAND OF METHOTREXATE TREATED RATS

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KEYWORDS

Methotrexate, L-carnitine, submandibular salivary glands, BCL-2, Caspase-3

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ABSTRACT

Introduction: Methotrexate is antimetabolite drug, that was used in the management of many malignant conditions. The main mechanism of MTX cytotoxicity involves a reduction in the effectiveness of the antioxidant enzyme defense system**.** So, antioxidants as L-carnitine may protect against oxidative stress induced cell damage by MTX. **Aim:** To evaluate the possible prophylactic and therapeutic effect of L-carnitine on the submandibular salivary gland of methotrexate treated rats, histological and immunohistochemical examination to localize caspase-3 and BCL-2 were carried out. **Materials and Methods:** Fifty male Wistar rats with an average of 150 -200 grams were divided into five groups, 10 rats for each group as following: Group 1: rats received no treatment. Group 2: rats received a single intraperitoneal injection of 20mg/kg MTX on day 5. Group 3: rats received: L-carnitine as intraperitoneal injection 500mg/kg per day & MTX as single intraperitoneal injection 20 mg/kg on day 5. Group 4: rats received 20 mg /kg of MTX as single intraperitoneal injection on day 5. & L-carnitine 500 mg / kg was given as treatment from day 5 till day 10**.** Group 5: rats received L-carnitine as intraperitoneal injection 500mg/kg per day. Subsequent to the experiment rats were euthanized and submandibular gland specimens were collected for histological and immunohistochemical examinations. All data was collected and statistically analyzed by the computer program SPSS software for windows version 26.0. **Results:** L-carnitine has a protective role against methotrexate cytotoxic effects and its usage as a prophylactic is more effective than its usage as a therapeutic drug. **Conclusion:** L-carnitine has a protective role against methotrexate cytotoxic effects and its use as a prophylactic drug was more effective than its use as a therapeutic one.

INTRODUCTION

Cancer is a complex disease that represents a major public health challenge worldwide. Its incidence varies in types across different populations. It is important to reveal that cancer cells are less specialized than normal cells and continue to divide uncontrollably. There are many biological capabilities that cancer cells have that are created by each tumor microenvironment in human tumors, such as sustained proliferation, evasion of growth inhibitors, evasion of apoptosis, immortality, induced angiogenesis, invasion and metastasis, genomic instability and inflammation [1, 2].

Chemotherapy is one of the major methods of tumor treatment, the damage degree differs according to the type, dosage and treatment duration[3]. As it has cytotoxic effects which are not selective for cancer cells but affect the normal tissues, so chemotherapeutic drugs are facing with great challenges such as poor delivery and potent side effects [4].

The most common side effects detected during chemotherapy treatment: mucositis (inflammation of the lining of the gastrointestinal tract), alopecia (hair loss) and myelosuppression (reduced production of blood cells, due to which is also immunosuppressive) [5].

Oxidative stress is a dynamic complex condition characterized by an imbalance between the reactive oxygen species (ROS) generation, the availability and action of antioxidants [6,7]. The escape from antioxidant mechanisms of ROS and their progressive accumulation activates lipid peroxidation mechanisms and damages the structure of proteins and DNA [8]. Oxidative stress is a factor in many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [9,10]. It is also believed to be a cornerstone of the pathophysiological mechanisms of drug-induced damage in many organs and tissues [11,12].

Methotrexate (MTX) is an antimetabolite that interferes with the metabolism of folic acid, known as amethopterin. It is one of various folic acid antagonists synthesized in the 1940s to treat malignant tumors and designed to inhibit dihydrofolate reductase (DHFR), an essential enzyme for the synthesis of purines and pyrimidines during cell proliferation [13,14].

Methotrexate (MTX) was widely used in the treatment of numerous malignant conditions in addition to some other diseases as rheumatoid arthritis, psoriasis, ectopic pregnancy, Crohn's disease, ulcerative colitis, cytopenia, infections, liver damage, mucocutaneous toxicity and hypersensitivity pneumonitis [15, 16].

Severe side effects were observed in patients treated with MTX, the extent of damage varying depending on the type and duration of treatment [3]. Common side involve multi-organ toxicity such as the gastrointestinal tract and liver, as well as immune disorders, inflammation or lymphoproliferation, and major toxic effects on the salivary glands [13]. Saliva and salivary glands are believed to be the main link between oral health and overall systemic health [17]. Because its use is limited by serious side effects, finding ways to reduce its side effects represents a significant scientific challenge [18,19].

One of the main mechanisms of MTX's cytotoxicity is to reduce the effectiveness of the antioxidant enzyme defense system, making cells vulnerable to reactive oxygen species (ROS). Therefore, antioxidant variants may protect against cellular damage caused by MTX-induced oxidative stress [20]. It is widely assumed that oxidative stress caused by formation of free radicals and oxidants is balanced by antioxidant defense mechanisms that eliminate reactive oxygen species and decrease oxidative cellular damage. [21].

Antioxidants taken as nutritional supplements may neutralize ROS stress and protect against apoptosis [22]. According to recent studies l-carnitine has antioxidant properties and can protect cells from toxic reactive oxygen species in some metabolic disorders[23]. Carnitine is considered an essential nutrient[24]. Carnitine (3-hydroxy-4-N-trimethyl-ammonio-butanoate) is a quaternary ammonium compound biosynthesized from lysine and methionine [25]. L-carnitine may accumulate in liver, skeletal muscle, heart, brain and testis [26].

L-carnitine is involved in fatty acids oxidation

and energy production, transports long-chain fatty acids into the mitochondria for oxidization, leading to energy production. It also transports toxic compounds out of this cellular organelle to avoid their accumulation. LC exerts its antioxidant effects by decreasing reactive oxygen species (ROS) and acts as a metal chelator to reduce free radical formation by preserving and conserving antioxidant enzyme levels [27].

The major regulatory role of LC in antioxidant processes was discussed in various organs like heart, colon, retina, and brain [28-30]. Cell protecting activity of LC against oxidative damage seen during neurodegenerative disorders such as in Parkinson's and Alzheimer's diseases. LC reduces oxidative stress significantly during aging. LC was found to enhance the activities of antioxidant enzymes, thereby decreasing the lipid peroxidation and formation of superoxide radicals in the heart of spontaneously hypertensive rats [31].

It is known that all chemotherapeutic agents as MTX may trigger apoptosis through oxidative stress [32]. Apoptosis can be one of the main causes of acute submandibular salivary gland injury after methotrexate treatment [33]. Caspase-3 is a member of the cysteine protease family and is responsible for cleaving key cellular proteins, leading to typical morphological changes in apoptotic cells [34]. In addition to BCL-2 (B-cell lymphoma 2) that is considered as an oncogene and one organizing member of the Bcl-2 family regulating proteinosis and is specifically deemed to be remarkable anti-apoptotic protein [17].

Studies revealed that MTX induces cytotoxicity in the submandibular salivary glands of rats [33,35,36]. Also L-carnitine was proved to have significant protective and antioxidant effects on certain tissues and organs such as the liver. [20,27,37,38].

Up till now, no published data as far as our knowledge, studied the effect of L-carnitine on submandibular salivary gland of methotrexate treated rats, so this study was designed to study histopathological changes that occurred in methotrexate treated submandibular salivary gland of rats and the protective and therapeutic effect of LC supplementation.

MATERIALS AND METHODS

Ethical consideration: The present research was an experimental study carried out on fifty male Wistar rats, conducted after the Research Ethics Committee (REC) approval of the Faculty of Dentistry, Suez Canal University with approval number (413/2021). Following the ethical guidelines of animal care.

Materials: Materials used in this study included Methotrexate mylan, Solution for injection, 50mg per 2 ml of solution, Haupt Pharma GmbH – Imported by: RAMCO, Germany. Lot no:5104 & L-carnitine Solution, 1gm/5ml SEDICO Pharmaceuticals, for: Arab Co. for Pharmaceutical & Medicinal Plants (MEPACO – MEDIFOOD) – Egypt. Lot no.: TNS105.

Methods:

Study design: The study was conducted on fifty male Wistar albino rats with an average of 150-200 grams body weight (according to sample size calculation). They were housed in the animal house, Faculty of Dentistry, Suez Canal university, where they were acclimated for 7 days before the beginning of treatment, they were kept in 12h/12h dark and light cycle with free access to food and water for 10 days.

Animals:

Sample Size Calculations

The study sample size was calculated according to **Charan and Biswas** [39] used the following equation:

$$N = \frac{N = (Z\alpha)^2 \times (SD)^2}{d^2}$$

N = Total sample size

Zα= Is Standard normal variate and its equal 1.96 at P< 0.05

SD = Standard diversion of variable

d = Absolute error or precision

Zα	SD	D
1.96	7.20	2

Total Sample size N =

$$\text{Total Sample size } N = \frac{(1.96)^2 \times (7.20)^2}{(2)^2}$$

= **49.787≈50** samples

The total sample size calculations revealed that a sample size should be **50**, and they were divided equally into five groups**.**

Animal grouping:

The animals were divided randomly into five groups, 10 rats for each group as follows: **Group 1** (negative control): 10 rats received no treatment. **Group 2** (MTX treated group):10 rats received a single intraperitoneal injection of 20mg/kg MTX[33.35] on day 5. **Group 3** (MTX with L-carnitine prophylactic group): 10 rats received L-carnitine as intraperitoneal injection 500mg/kg per day (days 1:10)[20] and MTX as single intraperitoneal injection 20 mg/kg on day 5. **Group 4** (MTX with L-carnitine therapeutic group): 10 rats received 20mg/kg of MTX as single intraperitoneal injection on day 5 & L-carnitine 500mg/kg was given as treatment from day 5 till day 10[20.33]. **Group 5 (**L-carnitine treated group): 10 rats received L-carnitine as intraperitoneal injection 500 mg / kg per day (days 1:10) [20]. After the experiment which lasted for 10 days, all rats were anesthetized with intraperitoneal dose of ketamine (10%) and xylazine (2%) mixture and were euthanized by overdose of ether [40].

Weight assessment:

The rats in each group were weighted before experiment (baseline weight) and after experiment (final weight).

Histological procedure:

Preparation and staining of specimens:

After submandibular salivary glands were excised bilaterally, specimens were fixed in 10% formalin buffered saline for 48 hours. Then dehydrated, cleared, infiltrated with molten paraffin wax and embedded in hard paraffin. Then sections of 3-5 microns obtained and prepared for:

1. Haematoxylin & eosin stain to study the general histological changes.
2. Immunohistochemical staining using rabbit monoclonal antibody (R&D Systems Catalog # MAB835) to cleaved Caspase-3 as cell apoptotic marker & mouse monoclonal antibody (R&D Systems Catalog # MAB8272) for localization of B-cell lymphoma-2 (BCL-2) as anti-apoptotic marker.

Immunohistochemical evaluation

Image analyzer computer system (Image J / Fiji 1.46) was used for:

Immunohistochemical assessment through measuring the optical density of immunostained cells and digitizing the slides under 400X objective magnification in each group.

The positive results for markers were indicated by cytoplasmic brown coloration with different intensities which were statistically analyzed and compared [41].

Statistical analysis

Data were collected, calculated, tabulated and statistically analyzed using the following statistical tests. A normality test (Shapiro-Wilk) was done to verify the normal distribution of the samples. Descriptive statistics were calculated in the form of Mean ± Standard deviation (SD). One way analysis of variance (ANOVAs) was used to compare between four groups. Tukey`s post hoc tests were used for pairwise comparisons. P value ≤ 0.05 is considered statistically significant. All analysis was done using the computer program SPSS software for windows version 26.0 (Statistical Package for Social Science, Armonk, NY: IBM Corp) at significant levels 0.05 (P- Value ≤0.05).

RESULTS

Mortality rate: The percentage of mortality of both control and treated groups was 0%.

Clinical results:

The rats in each group were weighted before experiment (baseline weight) and after experiment (final weight). Comparison between different groups at baseline and final weight were done (**Table 1**).

Histological Results: Group 1 (negative control): The submandibular salivary gland of the negative control group revealed normal gland structure. The gland was surrounded by a fibrous connective tissue capsule which sent out septa that divided the gland into lobes and lobules. Parenchymal elements which were invested in the reticular connective tissue stroma included collagen fibers, fibroblasts and other connective tissue cells embedded in the ground matrix, together with blood vessels, lymphatics and nerves. The gland revealed only one type of acini, serous acini. The serous acini consisted of pyramidal cells surrounding a narrow lumen with moderately basophilic cytoplasm and a round basally situated nuclei while the ducts appeared more acidophilic. The duct system consists of intercalated, granular convoluted tubules, striated and excretory ducts **(Figure 1-A).**

Group 2 (MTX treated Group) revealed marked degeneration and disorganization in the parenchymal elements including the acini and ducts compared to the controls with widening of connective tissue septa spaces between the lobes **(Figure 1-B, D)**. The serous acini showed shrinkage and degeneration with a lot of cytoplasmic vacuolization and pyknotic nuclei **(Figure 1-C)**. The granular convoluted tubule ducts showed loss of configuration, their cells showed reduction in number of granularity with deeply stained irregular nuclei and cytoplasmic vacuolization. The striated ducts showed shrinkage, cytoplasmic vacuolization and basal striations loss **(Figure 1-C)**. The excretory ducts showed loss of the pseudostratification of their lining. Most of ducts showed dilatation in their lumens with secretion stagnation with prominent increase in the interstitial connective tissue. Dilatation of blood vessels was detected and red blood cells engorgement **(Figure 1-E)**.

Group 3 (MTX with L-carnitine prophylactic group) showed marked improvement of their histological picture in comparison to MTX treated group. No widening of connective tissue septa spaces between the lobes was detected **(Figure 1-F)**. Acinar cells and granular convoluted tubules (GCTs) of submandibular glands of the prophylactic group rats showed normal texture with no or little cytoplasmic vacuolization **(Figure 1-G)**. The striated ducts regained their basal striations **(Figure 1-G)**. The excretory ducts showed pseudo stratification of their lining with no stagnations in lumens **(Figure 1-H)**.

Group 4 (MTX with L-carnitine therapeutic group) showed minimal improvement of their histological picture in comparison to MTX treated group. An increase in fibrous CT content surrounding ducts and interstitial connective tissue was sometimes recorded **(Figure 1-I)**. Few Acinar cells and ducts of submandibular glands of the therapeutic group rats showed almost normal architecture with little cytoplasmic vacuolization **(Figure 1-J)**. Shrunken acini and ducts still be noticed with minimal stagnation and cytoplasmic vacuolization. Some of striated ducts regained their basal striations but the granular convoluted tubules still sub normal **(Figure 1-J)**. Dilatation of blood vessels were still detected with RBCs engorgement **(Figure 1-K)**.

Group 5 (L-carnitine treated group) showed picture close to normal control group with no significant difference in collagen in trabeculae and around ducts in comparison to control group **(Figure 1-L)**.

Immunohistochemical results:

Negative controls of immunohistochemical staining: the primary antibody was substituted by the nonspecific serum of the same dilution as its respective antibody and procedure **(Figure 2-A)**.

a. **Caspase-3:** Negative control group of rat submandibular salivary glands showed mild cytoplasmic reaction of caspase-3 while MTX treated group showed strong cytoplasmic reaction of acini and ducts of caspase-3 **(Figure 2-B, C)**. In Group 3 (MTX with L-carnitine prophylactic group) ssubmandibular salivary glands showed negative cytoplasmic reaction of acini and moderate cytoplasmic reaction of ducts of caspase-3 while in Group 4 (MTX with L-carnitine therapeutic group) showed negative cytoplasmic reaction of acini and moderate to strong cytoplasmic reaction of ducts of caspase-3 **(Figure 2-D, E)**. Group 5 (L-carnitine treated group) submandibular salivary glands showed negative cytoplasmic reaction of caspase-3 **(Figure 2-F)**.

Pair wise comparison showed significant difference between all studied groups except G1 with G5, G2 with G4 and G3 with G4. The high value was recorded in G2 (131.77±3.62) followed by G4 (120.72±7.63) and G3 (106.69 ± 5.07) while G5 and G1 were the lowest (13.10 ±4.21) and (26.73 ±4.01) **(Table 2)**.

b. **BCL-2**: Negative control group of rat submandibular salivary glands showed mild cytoplasmic staining reaction of acini and strong cytoplasmic staining reaction of ducts of BCL-2 while Group 2 (MTX treated group) showed negative cytoplasmic staining reaction of BCL-2 **(Figure 2-G, H)**. In Group 3 (MTX with L-carnitine prophylactic group) submandibular salivary glands showed strong cytoplasmic staining reaction of acini and ducts while Group 4 (MTX with L-carnitine therapeutic group) showed negative cytoplasmic staining reaction of acini and strong cytoplasmic staining reaction of ducts of BCL-2 **(Figure 2-I, J)**. In Group

5 (L-carnitine treated group) submandibular salivary glands showed mild to moderate cytoplasmic staining reaction of acini and myoepethelial cells and strong cytoplasmic staining reaction of ducts of BCL-2 **(Figure 2-K)**.

Pair wise comparison showed significant difference between all studied groups except (G1) with (G3) and (G4). The high value was recorded in G5 (105.07±7.50) followed by G1 (101.03±2.736) and G3 (94.33 ± 4.70) while G2 and G4 were the lowest (55.95 ±4.69) and (75.94 ±4.70) **(Table 2)**.

Table (1) *Descriptive statistical analysis of baseline and final weight of all rats*.

	Base line weight	Final weight	% Change	Paired T- test	P value
	Mean ± SD	Mean ± SD			
G1	191.4±7.8 [a]	223.9±10.6 [a]	17.0	-24.81	<0.0001**
G2	191.3±7.3 [a]	161.7±9.4 [c]	-15.5	16.31	<0.0001**
G3	190.8±7.1 [a]	220.0±9.6 [a]	15.3	-13.24	<0.0001**
G4	189.4±6.7 [a]	209.4±9.1 [b]	10.6	-21.48	<0.0001**
G5	189.1±7.5 [a]	222.9±6.7 [a]	17.9	-18.17	<0.0001**
F test	0.221	82.113			
P value	0.925	<0.0001**			

Pair wise comparison		
	P value	**P value**
G1 Vs G2	1.00 ns	<0.0001**
G1 Vs G3	1.00 ns	1.000
G1 Vs G4	1.00 ns	0.010**
G1 Vs G5	1.00 ns	1.000
G2 Vs G3	1.00 ns	<0.0001**
G2 Vs G4	1.00 ns	<0.0001**
G2 Vs G5	1.00 ns	<0.0001**
G3 Vs G4	1.00 ns	0.131
G3 Vs G5	1.00 ns	1.000
G4 Vs G5	1.00 ns	0.019**

***; and different letters means significant Ns; means no-significant difference*

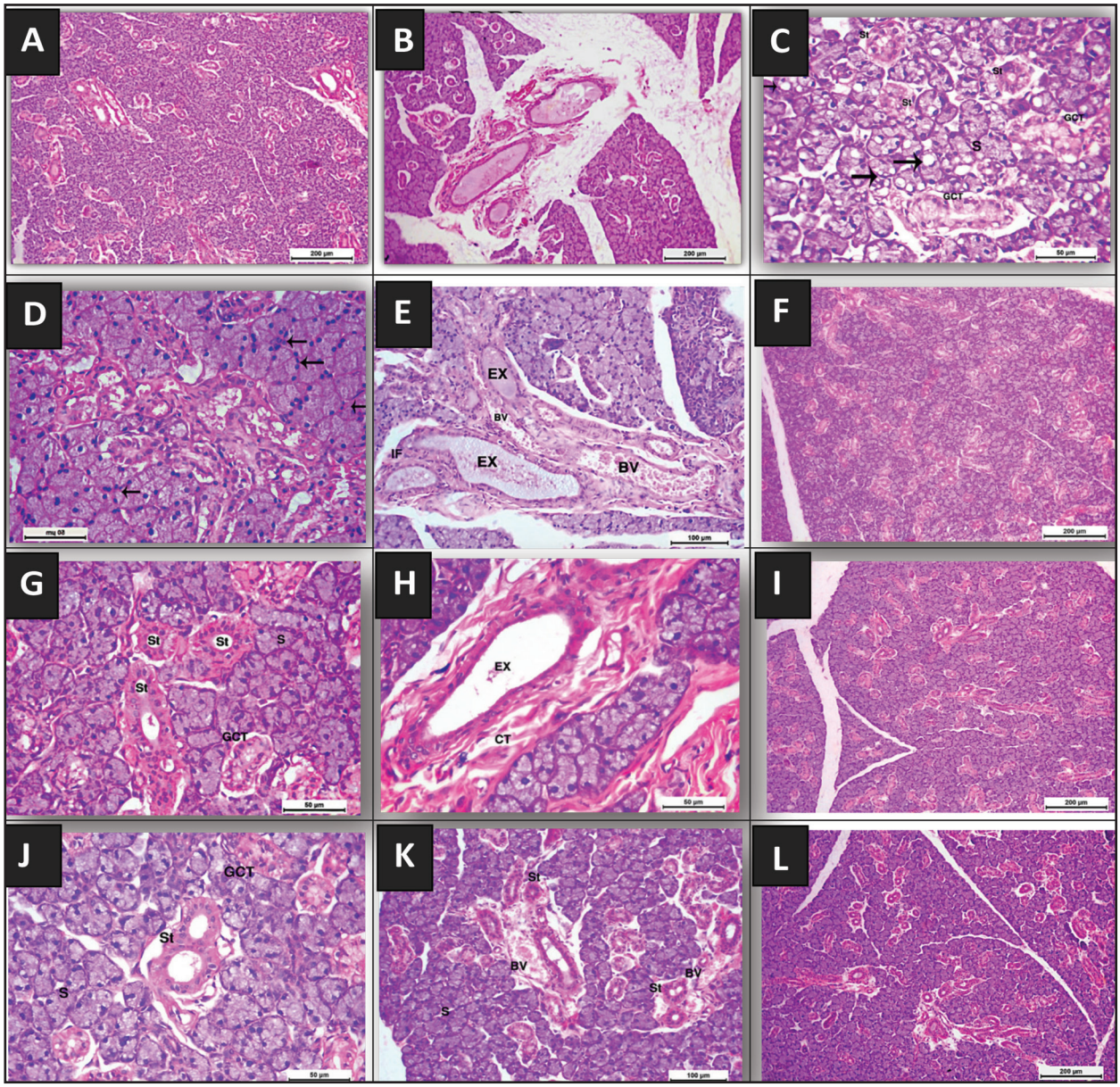

Fig. (1) Photomicrographs of H&E sections showing: A): Submandibular salivary glands of group 1 with normal architecture of the acini and the ducts. B): Submandibular salivary glands of group 2 showing reduction in size of parenchymal elements of the gland. C): Serous acini (S) with massive cytoplasmic vacuoles (arrows), GCTs showing loss of configuration with cytoplasmic vacuolization (GCT) and straited ducts showing loss of striations (St). D): Disorganization of parenchymal elements). E): Complete blockage of excretory ducts lumens and thinning of lining epithelium (EX), large dilated congested blood vessels (BV) and inflammatory cells infiltration (IF). F): Submandibular salivary glands of group 3 showing regularly organized acini and ducts. G): Densely packed serous acini(S), granular convoluted tubules still show degree of disconfiguration (GCT) and striated ducts with basal striations (St). H): Showing excretory duct with normal lining with no stagnation of its lumen (EX) and surrounding by fibrous connective tissue (CT). I): Group 4 showing regularly arranged acini and ducts. J): Showing closely packed serous acini (S), some striated ducts regained their basal striations (St), and granular convoluted tubule is still subnormal (GCT). K): Showing serous acini (S), dilated congested blood vessels (BV) and striated duct (St). L): Group 5 showing closely packed serous acini and typical ducts with normal connective tissue septs between lobes.

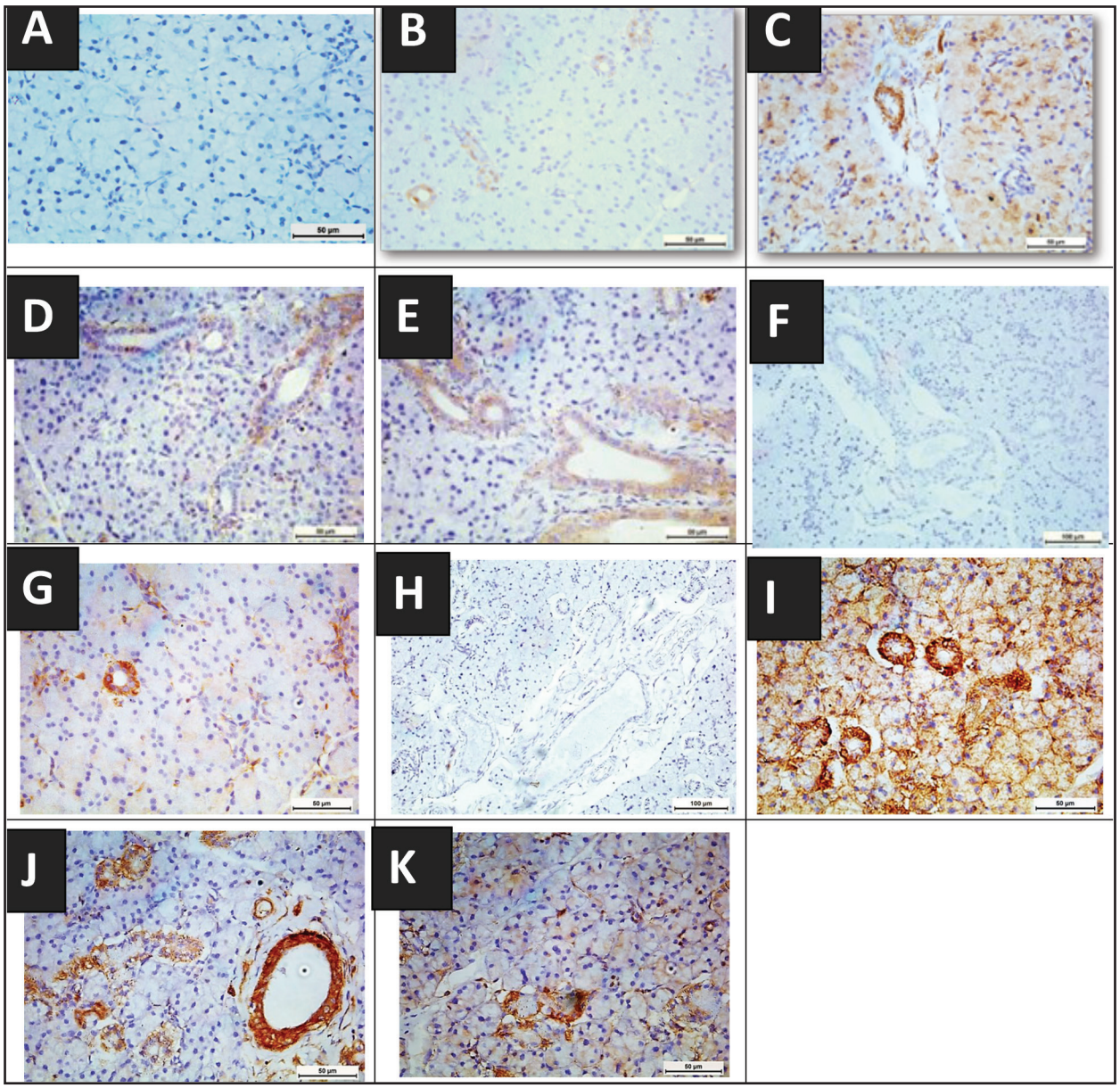

Fig. (2) A): A photomicrograph of rat submandibular salivary gland of negative control of immunohistochemical staining. B): Group 1 showing mild cytoplasmic staining reaction of caspase-3 of ducts. C): Group 2 showing strong cytoplasmic staining reaction of caspase-3of acini and ducts. D): Group 3 showing negative cytoplasmic reaction of caspase-3 of acini and moderate cytoplasmic reaction of ducts. E): Group 4 showing negative cytoplasmic reaction of caspase-3 of acini and moderate to strong cytoplasmic staining reaction of ducts. F): Group 5 showing negative cytoplasmic staining reaction of caspase-3 of ducts. G): Group 1 showing mild cytoplasmic reaction of BCL-2 of acini and strong cytoplasmic staining reaction of ducts. H): Group 2 showing negative cytoplasmic staining reaction of BCL-2. I): Group 3 showing strong cytoplasmic staining reaction of BCL-2 of ducts and acini. J): Group 4 showing negative cytoplasmic staining reaction of BCL-2 of acini and strong cytoplasmic staining reaction of ducts. K): Group 5 showing moderate cytoplasmic staining reaction of BCL-2 of acini and strong cytoplasmic staining reaction of myoepithelial cells.

Table (2) *pair wise comparison using Tukey's post hoc for caspase -3 marker*

	Mean difference	P values
G1 Vs G2	-105.04	<0.00001**
G1 Vs G3	-79.96	<0.00001**
G1 Vs G4	-93.99	<0.00001**
G1 Vs G5	13.63	0.0855
G2 Vs G3	25.08	0.0013**
G2 Vs G4	11.05	0.2452
G2 Vs G5	118.67	<0.00001**
G3-Vs G4	-14.03	0.0726
G3-Vs G5	93.59	<0.00001**
G4-Vs G5	107.62	<0.00001**

****, means significant difference at P<0.05 using Tukey's post hoc**

Pair wise comparison using Tukey's post hoc for BCL-2 marker

	Mean difference	P values
G1 Vs G2	45.08	0.000005**
G1 Vs G3	6.70	0.993
G1 Vs G4	25.09	0.000825**
G1 Vs G5	-4.03	0.9781
G2 Vs G3	-38.38	0.000020**
G2 Vs G4	-19.99	0.004869**
G2 Vs G5	-49.11	0.000002**
G3-Vs G4	18.39	0.008937**
G3-Vs G5	-10.73	0.215558
G4-Vs G5	-29.12	0.000237**

****, means significant difference at P<0.05 using Tukey's post hoc**

DISCUSSION

Oral mucositis, a main chemotherapy side-effect, badly affects patients' nutrition and life quality during and after treatment. Salivary dysfunction was reported secondary to chemotherapy and exaggerate mucositis induced by chemotherapy [36]. The present study attempted to detect the possible degenerative effect of methotrexate on submandibular salivary gland of rats and compare between prophylactic and therapeutic effect of L-carnitine on submandibular gland of methotrexate treated rats.

Clinical results of group 1 (negative control) showed an increase in weight of 16.9% which was near enough to group 5 (L-carnitine treated group) of 17.8%. In group 2 (MTX treated group) showed significant weight loss of 15.4% in contrary to group 3 (MTX with L-carnitine prophylactic group) & 4 (MTX with L-carnitine therapeutic group) which showed increase in body weight of 15.3% and 11% respectively. **Al-Moula *et al,*** [4] who explained loss of weight due to loss of appetite due to anticancer therapy.

The histological results of group 1 (negative control) which didn't receive any treatment showed normal gland architecture and consisted of serous acini only was in agreement with **Miclaus *et al,*** [42]. However this result disagreed with that of **Tamborrini *et al,*** [43] in which they revealed that it was mixed salivary gland. Also histological examination of the submandibular salivary glands of group 5 (L-carnitine treated group) showed picture close to normal negative control group with no significant difference in collagen compared to negative control group. These findings were explained by **Koohpeyma *et al***, [44] who proved the effect of L-carnitine as antioxidant which acts as potential scavenger of reactive oxygen species and protect tissues from damage.

Light microscopic examination of group 2 (MTX treated group) showed degeneration and disorganization in the parenchymal elements including the acini and ducts with widening of connective tissue septa spaces between the lobes. These finding were agreed with many studies reported that MTX induced cytotoxicity in salivary glands of rats, **Al-Refai *et al,*** [33] examined the cytotoxic effects of single dose of methotrexate on rat's submandibular gland and revealed that the severity of pathological changes increased as chemotherapeutic doses increased. Fibrosis was more obvious and this was supported by **Fawzy *et al***, [35] who reported that MTX-induced salivary glands degeneration occurred one week after MTX injection. Cytotoxic effect appeared in all glands submandibular, parotid and sublingual salivary glands and showed an increase in the collagen thickness in trabeculae and around ducts. This could be explained by oxidative stress and inflammation as reported by **Braicu *et al,*** [45] who stated that oxidative stress could release inflammatory cytokines, cells differentiation to myofibroblasts and deposition of extracellular matrix components. On the other hand, **Al-Refai *et al***, [33] explained the increase in collagen by acinar and ductal cell death followed by replacement of connective tissue.

Serous acini showed shrinkage with a lot of cytoplasmic vacuolization which agreed with **El-Agamy *et al,*** [46]. **Ali *et al***, [47] explained this vacuolization as a result of fatty degeneration and lipid droplets accumulation in the cytoplasm. These droplets might unite together forming a large vacuole and consequently lipid degeneration and death of cells. But according to **Lombaert *et al***, [48] reported that these vacuoles might be due to swelling of mitochondria in acini and ducts.

Furthermore, striated ducts could not be distinguished clearly at group 2 (MTX treated group) that was detected by reduction of epithelial height and basal infoldings loss. These results were reported by **Al-Refai *et al,*** [33] who explained that MTX induced degeneration of these duct cells. Excretory ducts showed dilatation in their lumens with secretion stagnation. **Moheb *et al,*** [49] reported that this ducts dilatation suggested the pathologic effect of MTX on myoepithelial cells embracing them with failure of secretion expelling into the oral cavity because of glandular dysfunction leading to xerostomia.

Areas of cellular infiltration were detected around the ducts. This was in agreement with **Cure *et al,*** [50] who attributed the cell infiltration in this study to the effects of oxidative stress because ROS damage connective tissue as well as DNA and cell membranes leads to stimulation of macrophages, neutrophil infiltration and release of proinflammatory cytokine. Dilatation of blood vessels and red blood cells engorgement were detected. **Zahawi** [51] also reported marked vascular dilatation and congestion appeared in SMG of rabbits treated by MTX. Such dilatation and congestion might be due to inflammatory response associated with MTX treatment which increase trans endothelial permeability as reported by **Garipardic *et al***,[52]**.**

Group 3 (MTX with L-carnitine prophylactic group) showed acini and ducts almost with normal texture with no or little cytoplasmic vacuolization. No widening of connective tissue septa spaces between the lobes was detected. Normal blood vessels were detected. These findings were supported by **Khatab *et al,*** [53] **w**ho evaluated the protective effects of melatonin and L-carnitine against toxicity of MTX in isolated rat hepatocytes by pre-incubation of hepatocytes with melatonin and LC 30 min prior to intoxication with MTX. LC prevented hepatocytotoxicity by their antioxidative, anti-inflammatory and antiapoptotic effects.

In group 4 (MTX with L-carnitine therapeutic group) few acinar cells and ducts showed almost normal architecture. However shrunken acini and ducts still be noticed with minimal stagnation. Dilatation of blood vessels were still detected with RBCs engorgement. These findings were in agreement with **El Ghamrawy** [54] who mentioned that apoptotic changes in parotid gland caused by liquid diet prevented by antioxidant properties of LC. The parotid gland of the group on liquid diet supplemented with LC had normally eosinophilic cytoplasm with few vacuoles in their cells of acini. Also the improvement exerted by LC due to its antioxidant property supports the oxidative process rather than lipid degeneration. As a scavenger of reactive oxygen species, it prevents beta-oxidation degradation of fatty acids in mitochondria and protects damaged tissues. A notable improvement in vacuole-free cytoplasm and cell regeneration was observed in the LC-supplemented group with their abundant secretory granules.

According to this study usage of L-carnitine for prophylaxis may have more favorable protective effect against cytotoxicity of MTX in the submandibular salivary glands of rats than its therapeutic effect. Many Studies revealed that prophylactic treatment has better effect than therapeutic treatment which agreed with this current study [54].

Immunohistochemical staining was performed using Caspase-3 and BCL-2, Caspase-3 is responsible for the cleavage of key cellular proteins, which leads to typical morphological changes in cells undergoing apoptosis [41]. In addition to BCL-2 is specifically deemed to be remarkable anti-apoptotic protein [17].

Immunohistochemical results of group 1 (negative control group) of rat submandibular salivary glands showed negative to mild cytoplasmic reaction of caspase-3. This finding was in accordance with **Elmansy and Hegazy** [41] who reported mild cytoplasmic reaction in duct and acini of submandibular salivary gland of negative control group as it is apoptotic marker.

Group 5 (L-carnitine treated group) of rat submandibular salivary glands showed negative cytoplasmic reaction of caspase-3 and this finding was in agreement with **Kelek *et al***, [38] who reported a decrease in caspase-3 activity in L-carnitine supplemented rats compared to control. They explained that L-carnitine has a stabilizing activity on the outer mitochondrial membrane, which can prevent the efflux of cytochrome C into the cytosol and this is a critical point in the apoptotic cascade.

Immunohistochemical results of group 2 (MTX treated group) in our study showed significant increase in the apoptotic activity of caspase-3 compared to group 1 (negative control), That was supported by **Fawzy *et al,*** [35] who revealed remarkable increase in caspase-3 reaction in MTX treated group in comparison with control group in all salivary glands. This result was also supported by **Al-Moula *et al,*** [3] as regards Caspase-3 immunoreactivity in MTX group revealed positive cytoplasmic Caspase-3 immunoreaction in cells of acini and ducts in all salivary glands. This was explained by disruption of protein synthesis through depletion of foliate co-factors and formation of cytolysosome which considers evidence of apoptosis.

In group 3 (MTX with L-carnitine prophylactic group) remarkable decrease of caspase-3 expression in comparison with group 2 (MTX treated group). In group 4 (MTX with L-carnitine therapeutic group) caspase-3 expression showed statistically significant decrease in comparison with group 2 (MTX treated group). So, there was a decrease in caspase-3 expression of group 3 in comparison with group 4.

The immunohistochemical assessment of caspase-3 in the present study agreed with previous studies that also showed that L-carnitine suppressed caspase-3 [55.56]. Also **ElGhamrawy** [54] explained that LC supplementation can prevent apoptotic changes in parotid gland caused by liquid diet by its antioxidant properties and showed marked decrease in caspase-3 expression.

Immunohistochemical results of BCL-2 of group 1 (negative control group) of rat submandibular salivary glands showed mild cytoplasmic staining reaction of acini and strong cytoplasmic staining reaction of ducts which was in agreement with **El Emam *et al,*** [57].

Group 5 (L-carnitine treated group) showed mild cytoplasmic staining reaction of acini and myoepithelial cells and strong cytoplasmic staining reaction of ducts of BCL-2. This finding was in agreement with **Kelek *et al,*** [38] who reported a slight increase in cytoplasmic staining of L-carnitine treated group compared to negative control group. **Tousson *et al***, [58] explained this finding as that L-carnitine supplementation in rats can increase Bcl-2 and decrease Bax protein compared with non-treated controls.

Immunohistochemical results of group 2 (MTX treated group) in our study showed significant decrease in the antiapoptotic activity of BCL-2 in the gland in comparison with group 1 (negative control). This agreed with **Al-Refai *et al,*** [33] who reported negative or decreased cytoplasmic reaction of Bcl-2 in submandibular salivary gland of MTX treated group in comparison with negative control group. This could be explained by the fact that methotrexate consumption is associated with an increased accumulation of reactive oxygen species (ROS), leading to more oxidative stresses inside the cell, promoting apoptosis by increasing the proapoptotic promoting genes (Bax and caspases) while decreasing Bcl-2 production [57].

By the administration of L-carnitine statistically significant increasing in the antiapoptotic activity BCL-2 in relation with the methotrexate treated group, in group 3 (MTX with L-carnitine prophylactic group) remarkable increase of expression of BCL-2 in comparison with group 2 (MTX treated group) as showed strong cytoplasmic staining reaction of ducts and acini. In group 4 (MTX with L-carnitine therapeutic group) BCL-2 expression showed statistically significant increase in comparison with group 2 (MTX treated group) but showed negative cytoplasmic staining reaction of acini and strong cytoplasmic staining reaction of ducts. So, there was statistically significant increase in BCL-2 expression of group 3 compared with group 4.

These results explained by **Kelek *et al***, [38] that L-carnitine enhanced Bcl-2 expression and decreased Bax expression so it may inhibit apoptosis. Due to the positive consequences of L-carnitine, it can be used as a prophylactic treatment. Our results support the hypothesis that the tissue protective influence of L-carnitine on apoptosis is mediated by regulating the expression of Bcl-2.

Up to sum, histological and immunohistochemical results of the present study showed that the usage of L-carnitine for prophylaxis may have more favorable protective effect against MTX cytotoxicity in the rats submandibular salivary glands than its therapeutic effect.

CONCLUSION

Methotrexate chemotherapeutic drug has major cytotoxic effects on submandibular salivary gland of albino rats. L-carnitine has a protective role against methotrexate cytotoxic effects and its use as a prophylactic (before MTX injection) is more effective than its use as a therapeutic drug (after MTX injection).

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