

## Protective Effects of L-Carnitine on Cisplatin Induced Toxicity In rat Parotid Salivary Glands

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**Abstract: Background:** Cisplatin is one of the most effective chemotherapeutic drugs. However, it has severe side effects that limit its use. Acetyl L- Carnitine is a well known neuroprotective agent. It also has antioxidative as well as powerful antiapoptotic properties on various cell types. **Aim:** The purpose of this study was to evaluate the protective effects of acetyl L-Carnitine on Cisplatin induced cytotoxicity on rat parotid salivary glands. **Methods:** Thirty male albino rats (250-300 grams) were divided equally into three groups. Group I (control group) was administrated saline. Group II (Cisplatin group) received cisplatin injection. Group III (Cisplatin & L- Carnitine group) was administrated L- Carnitine prior to cisplatin injection. Rats were sacrificed after 3 days of cisplatin/ saline administration. The parotid salivary glands were dissected out and prepared for histological and immunohistochemical examinations. **Results:** Light microscopic examination of cisplatin group revealed enlargement and deformity of the secretory portions with numerous intracellular vacuoles. Secretory cells revealed deeply stained atrophied nuclei. The excretory ducts appeared dilated with degenerated epithelial lining. Widening of the connective tissue septa with chronic inflammatory cells infiltration was also detected. Some secretory cells and intralobular ducts were completely degenerated leaving large vacuoles. While L- Carnitine treated group revealed well defined serous acini having distinct outline and lined by pyramidal cells with rounded basophilic nuclei. Well formed striated ducts were also detected. There were numerous dilated blood vessels engorged with red blood cells. Immunohistochemical examination of Bax protein expression of cisplatin group showed significant increase in Bax positive immunoreactivity indicating apoptotic changes while L- Carnitine treated group revealed expression of Bax protein that statistically having no significant difference with control group. **Conclusion:** Administration of acetyle L- Carnitine produced a protective effect against cytotoxic and apoptotic changes induced by cisplatin treatment in rat parotid salivary glands.

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### 1. Introduction

Chemotherapy is one of the most widely used interventions for treatment of cancer. Cisplatin (cis-diammine dichloro platinum II, CDDP) is a potent and an effective chemotherapeutic agent used to treat various types of cancer as testis, bladder, head and neck, lung, and ovarian cancers. The clinical use of cisplatin is limited due to severe side effects. It produced a high incidence of toxicities as nephrotoxicity, gastrointestinal toxicity, neurotoxicity and ototoxicity<sup>(1,2)</sup>. Chemotherapy also caused different oral complains including mucositis (stomatitis); xerostomia (dry mouth); bacterial, fungal, or viral infection (particularly in neutropenic patients); dental caries and loss of taste<sup>(3)</sup>.

The cytotoxic (anticancer) effects of CDDP are due to coordinative bonds between the atom of platinum and DNA of the cells leading to formation intrastrand DNA crosslinks. This interaction of cisplatin with nuclear DNA may have important cellular effects contributing to apoptosis<sup>(4- 6)</sup>. Cisplatin also may directly lead to the generation of

reactive oxygen species or may induce the release of reactive oxygen molecules normally sequestered within mitochondria that may trigger several mechanisms of apoptosis. Reactive oxygen species mediated damage could occur as consequence of antioxidant depletion and increased lipid peroxidation<sup>(7)</sup>. Thus; several strategies have been suggested to prevent the oxidative stress-induced apoptosis of the tissues that have been exposed to cisplatin. Prevention of the formation of reactive oxygen species either by binding the toxin or reversing the binding of the toxin or inhibition of lipid peroxidation. Addition of exogenous free-radical scavengers or antioxidant enzymes as well as inhibitors of caspases and gene therapy to up regulate antiapoptotic gene products might be also useful<sup>(8, 9)</sup>. There are reports that antioxidants such as N,N'-diphenyl-1,4-phenylenediamine prevented the increases in content of lipid peroxides and nephrotoxicity induced by cisplatin<sup>(10)</sup>.

Acetyl-L-Carnitine (ALC) is a member of the family of carnitines, a group of natural compounds

that have an essential role in intermediary metabolism and facilitates the entry and exit of essential fatty acids from the mitochondria<sup>(11-14)</sup>. It also has a direct or indirect antioxidant activity. Different studies suggested an efficient role of ALC against cisplatin cytotoxicity without interfering with its antitumor efficacy. Additionally, ALC is safe and extremely well tolerated compound used in different range of clinical conditions<sup>(15)</sup>.

Chemotherapeutic drugs had many side effects so protection against their cytotoxicity is necessary. Therefore, the aim of this study was to evaluate whether L-Carnitine had a protective effect on cisplatin induced cytotoxicity in rat parotid salivary glands both histologically and immunohistochemically.

## 2. Material and Methods:

Thirty healthy adult male albino rats weighing 250 -300 grams were used in this study. They were kept on normal diet and water. The animals were divided into three main groups (10 rats each) as follows:

### Group I (Control group):

The rats received a single intraperitoneal injection of isotonic saline.

### Group II (Cisplatin group):

The rats received a single intraperitoneal injection of 7mg/ Kg b.wt. Cisplatin<sup>®</sup> (MERCK generiques- France)<sup>(16)</sup>.

### Group III (Cisplatin & L- Carnitine group):

The rats received a single intraperitoneal injection of 500 mg/kg L-Carnitine<sup>®</sup> (MEPACO-Egypt) before the intraperitoneal injection of 7mg/Kg Cisplatin<sup>®</sup> (MERCK generiques- France)<sup>(16)</sup>.

After one week, the rats were sacrificed by carbon dioxide inhalation. The parotid salivary glands were dissected out and cleaned rapidly of any adherent connective tissue. Then the parotid glands were fixed immediately in 10% calcium formol for 12 hours, washed by tap water, dehydrated in ascending grades of ethyl alcohol, cleared in xylol and embedded in paraffin wax. Then:

**I-** Sections of 6-7M were obtained and mounted on clean glass slides and stained with Haematoxylin and Eosin stain for light microscopic examination.

**II-** 5um thick sections were cut and mounted on poly-L-lysine coated glass slides and prepared for Bax immunohistochemical staining for detection of apoptotic changes in parotid glands.

### Immunohistochemical procedure:

The sections were deparaffinized, rehydrated in graded ethanol and phosphate buffer saline (PBS).

The deparaffinized sections were incubated in 3% hydrogen peroxide at room temperature for 10 min to inhibit the activity of endogenous peroxidase, wash with PBS, and non specific protein binding sites blocked with 10% rabbit serum at 37°C for 10 min. The primary antibody used was polyclonal rabbit anti- human Bax (Dako, Glostrup, Denmark. code No. A3533). The tissue sections were incubated with the primary antibody overnight in moist chamber at 4°C then rinsed with PBS, 3times, 2min each. The sections were labeled with a streptavidin- biotin method using Dako- LAB vision (Dako, Glostrup, Denmark). After washing with PBS, the tissue sections were visualized with a freshly prepared 0.02% w/v 3, 3 diaminobenzidine -HCl (DAB) until optimal staining was obtained. The slides were washed in three changes of PBS for 10 min each. Then slides were immersed in a bath of Mayer's haematoxylin for 1-5 min, slides were rinsed, dehydrated, cleared and examined.

### The immunostained sections were examined using:

**a) Ordinary light microscope** to assess the prevalence of Bax positive immunoreactivity in the parotid salivary glands.

**b) Image analyzer computer system** was used to assess the optical density of Bax positive cells and the intensity of the immunostaining. The image analysis was performed using a computer (software Leica Quin500) consisting of color video camera, color monitor, CBU of IBM personal computer connected to the microscope. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. The intensity of the reactions within the cells was measured by the optical density in 10 small measuring fields in each specimen using a magnification of 400. After grey calibration, the image is transformed into a grey delineated image to choose the areas exhibiting positive reactivity with accumulation of all grades of reactivity (i.e. minimum, maximum and median grey). Positive areas were masked by a blue binary color. Mean values were obtained for each case (Fig.1).

### Statistical analysis:

**Paired Student's t-Test** was used to compare the mean % values of Bax immunoreactivity between control group and cisplatin group as well as between control group and cisplatin and L-carnitine treated group. A p-value  $p < 0.01$  was considered significant.

## 3. Results

### I-Light microscopic results:

#### Group I (Control group):

The Light microscopic examination of the rat parotid glands of control group showed pure serous acini and intercalated ducts in between. The serous acini were uniform in shape, having narrow lumen, basophilic cytoplasm and lined by pyramidal secretory cells having rounded basophilic nuclei. The intercalated ducts were hardly detected as they were very small in size and compressed in between the serous acini. They were lined by small cuboidal cells having central rounded nuclei. Connective tissue septa that divided the gland into lobes and lobules were also detected (Fig. 2).

#### Group II (Cisplatin group):

Histological examination of the parotid glands of cisplatin group revealed enlargement of the serous acini with abnormal architecture and ill defined outline. The lining cells of the serous acini were indistinct and showed numerous intracellular vacuoles. The excretory ducts appeared dilated, having wide lumen and degenerated thin epithelial lining (Figs. 3 & 4). The serous cells showed deeply stained atrophied nuclei. Aggregations of eosinophilic material in between the secretory portions were identified (Fig. 5). The connective tissue septa of the parotid glands affected by cisplatin showed widening and increased fibrosis with chronic inflammatory cells infiltration as lymphocytes and mast cells (Fig.6). Some of the parotid glands lobes revealed complete degeneration of their secretory elements as well as glandular elements leaving large empty vacuoles surrounded by deep infiltration of chronic inflammatory cells (Fig.7).

#### Group III (Cisplatin & L- Carnitine group):

Histological examination of the parotid glands of L- Carnitine treated group revealed well defined serous acini having distinct outline and lined by pyramidal cells with rounded basophilic nuclei. Well formed striated ducts were also detected. The connective tissue septa appeared thin. There were numerous dilated blood vessels engorged with red blood cells (Figs.8 & 9).

## II- Immunohistochemical results:

#### Control group:

Immunohistochemical examination of Bax protein in rat parotid salivary glands of control group revealed slight Bax immunoreactivity in the secretory portions and duct system (Fig. 10a).

#### Group II (Cisplatin group):

Immunohistochemical examination of Bax protein in rat parotid salivary glands of cisplatin group showed intense Bax positive immunoreactivity in the secretory portions as well as in the duct system (Fig. 10b).

#### Group III (Cisplatin & L- Carnitine group):

Immunohistochemical examination of Bax protein in rat parotid salivary glands of L- Carnitine treated group revealed slight Bax immunoreactivity in the secretory portions and duct system (Fig. 10c). Statistical analysis using Paired Student's t-Test to compare between different groups showed, a significant increase in the mean optical density of the immunoreactivity of Bax protein in cisplatin group compared with control group (Table I). L- Carnitine treated group showed no significant difference in mean optical density of the immunoreactivity of Bax protein compared with control group (Table II). While it produced a significant decrease in the mean optical density of the immunoreactivity of Bax protein compared with cisplatin group (Histogram I).

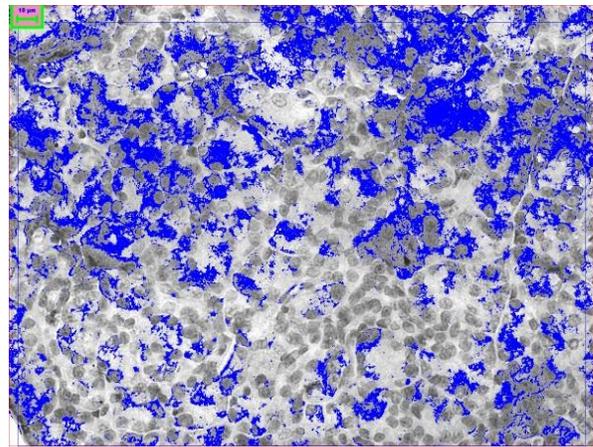


Fig. (1): A copy of display seen on the screen of the image analyzer showing the optical density of Bax immuno-expression after being masked by blue binary color.

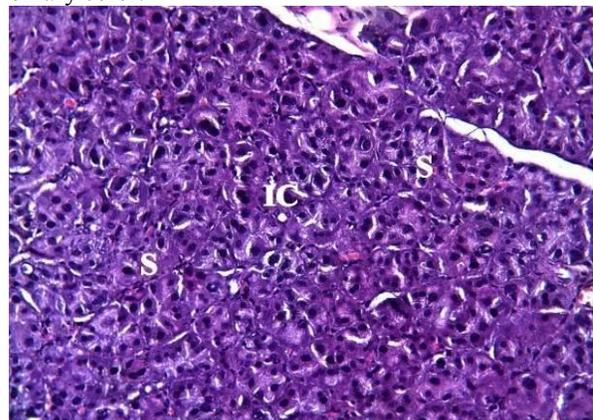
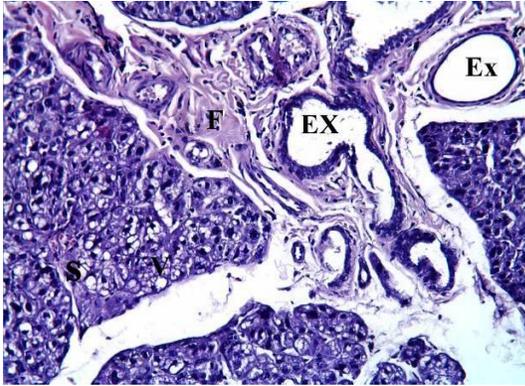
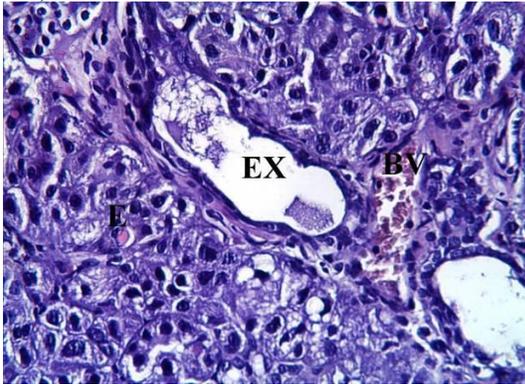


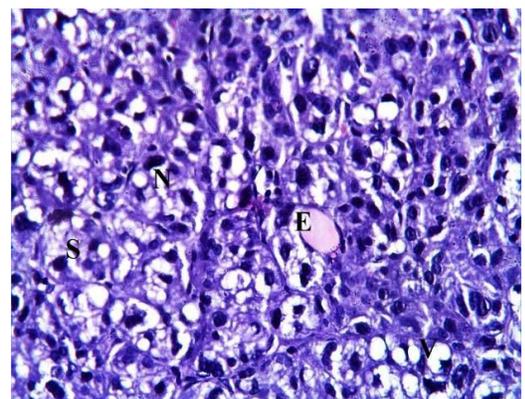
Fig. (2): A photomicrograph of rat parotid gland of control group showing the normal architecture of pure serous acini (S) and intercalated ducts in between (IC) . (H & E Orig.mag. X 200).



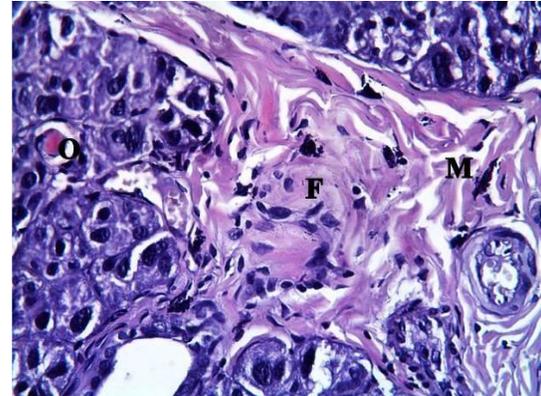
**Fig. (3):** A photomicrograph of parotid gland of cisplatin group showing enlarged serous acini with ill defined boundaries (S), numerous intracellular vacuoles (v) degenerated excretory ducts with thin epithelial lining (EX) with thin epithelial lining (EX) and extensive fibrosis (F) (H & E Orig.mag. X 200)



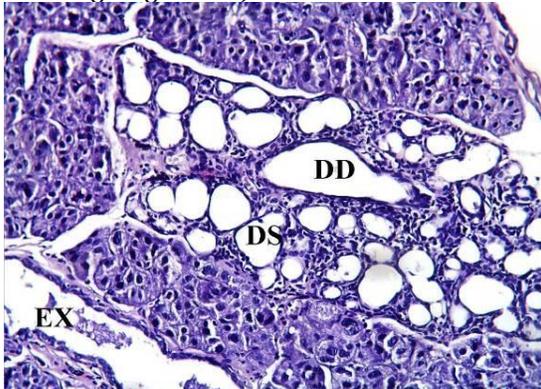
**Fig. (4):** A photomicrograph of parotid gland of cisplatin group showing dilated excretory ducts with thin epithelial lining (EX) and congested blood vessels (BV) (H & E Orig.mag. X 200).



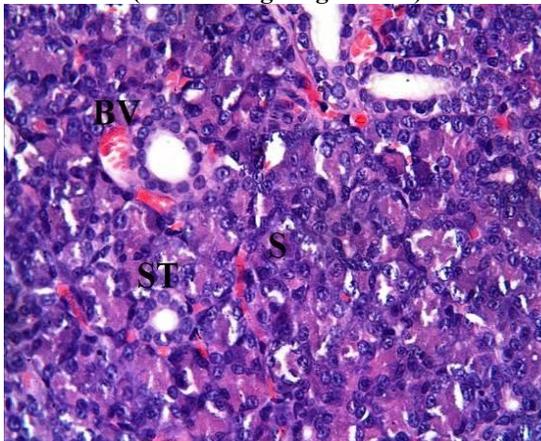
**Fig. (5):** A photomicrograph of parotid gland of cisplatin group showing enlarged serous acini with abnormal architecture (S), large eosinophilic material (E), numerous intracellular vacuoles (V) and deeply stained atrophied nuclei (N) (H & E Orig.mag. X 400).



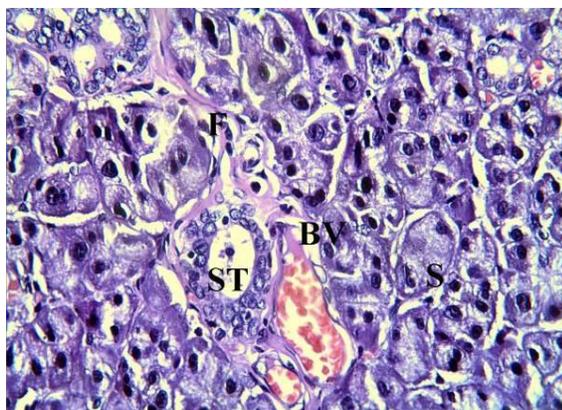
**Fig. (6):** A photomicrograph of parotid gland of cisplatin group showing extensive fibrosis (F) containing some lymphocytes and mast cells (M). (H & E Orig.mag. X 400)



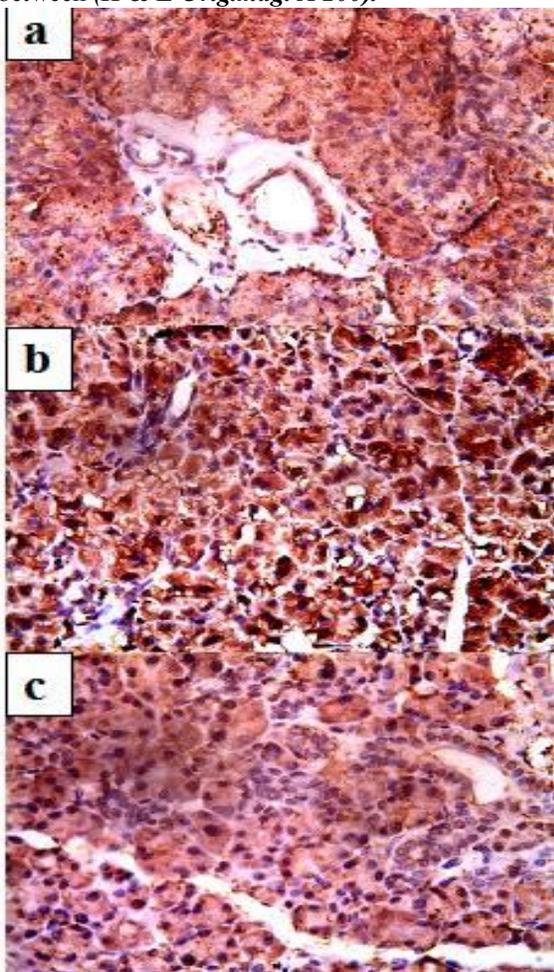
**Fig. (7):** A photomicrograph of parotid gland of cisplatin group showing numerous completely degenerated acini (DS), completely degenerated ducts (DD) surrounded by deep chronic inflammatory cells infiltration. (H & E Orig.mag. X 200)



**Fig. (8):** A photomicrograph of rat parotid gland of L-carnitine treated group showing pure serous acini (S), striated ducts (ST) and numerous dilated blood vessels in between (H & E Orig.mag. X 200).



**Fig. (9):** A photomicrograph of rat parotid gland of L-carnitine treated group showing well defined serous acini (S), striated ducts (ST), thin connective tissue septa (F) and numerous dilated blood vessels in between (*H & E Orig.mag. X 200*).



**Fig. (10):** A photomicrograph of rat parotid glands showing Bax immunoreactivity in the acinar and duct cells of (a) control group, (b) cisplatin group and (c) cisplatin and L-carnitine treated group (*Bax Orig.mag. X 200*).

**Table I: Difference in mean Bax optical density between control and cisplatin groups using Paired Student's t-Test**

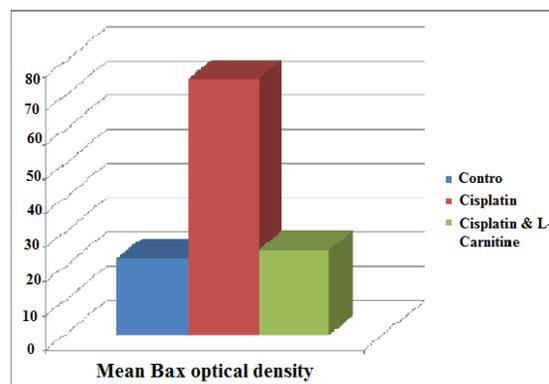
Group	Optical Density		
	M ±SD	t-Value	p-Value
Control	22.5460±0.3267	21.9785	0.0001**
Cisplatin	75.0080±0.3377		

\*\*Significant difference, (p<0.005).

**Table II: Difference in mean Bax optical density between control and cisplatin & L- carnitine treated groups using Paired Student's t-Test**

Group	Optical Density		
	M ±SD	t-Value	p-Value
Control	22.5460±0.3267	0.7596	0.4693
Cisplatin & L-carnitine	24.8460±0.1796		

No significant difference, (p>0.005).



**Histogram I: Represents Difference in mean Bax optical density between different groups.**

**4. Discussion:**

Cisplatin is a widely used and effective chemotherapeutic agent that binds to and alkylates DNA and triggers transcription inhibition, cell cycle arrest, and apoptosis<sup>(17, 18)</sup>. In addition, cisplatin generates reactive oxygen species which are known as one of the pathogenic intermediates following chemotherapy<sup>(19)</sup>. In the present study cisplatin injection altered the histological structure of parotid glands. Histological examination of the parotid glands of cisplatin group revealed enlargement of the serous acini with abnormal architecture and indistinct cell boundaries. There were numerous intracellular vacuoles with aggregations of eosinophilic material in between the secretory portions. These findings were in accordance with those found on the submandibular salivary glands due to intraperitoneal injection of cisplatin<sup>(20)</sup>. The enlargement of the serous acini might be due to glandular dysfunction and impaired exocytosis leading to accumulation of the salivary secretion in the acini followed by its swelling and enlargement.

In this study, numerous intracytoplasmic vacuoles were detected. These intracytoplasmic vacuoles might be attributed to intracytoplasmic degenerative changes within the acinar cells or due fusion of the secretory granules. This finding agrees with previous study, as cisplatin injection produced fusion of the secretory granules together on the first day after injection and large intracellular vacuoles were formed. Fusion of the secretory granules became more apparent on the third day. The content of the secretory granules was detected as central part of protein and peripheral rim of sialomucin. Aggregation of the secretory granules within the secretory cells was thought to be due to enhanced salivation in order to eliminate cisplatin shortly after infusion<sup>(20)</sup>.

In the present study, the nuclei of the secretory cells of the parotid glands showed deeply stained atrophied nuclei. These findings coincide with a previous study on the liver. As cisplatin injection produced liver injury manifested as focal necrotic changes and portal inflammation<sup>(16)</sup>. These nuclear changes were thought to be due to the mechanism of action of cisplatin. Absorption of cisplatin into the nucleus DNA, binding two adjacent guanines in the same DNA strand causing an inhibition of DNA synthesis and cell death<sup>(21)</sup>. In addition, Programmed cell death or apoptosis has been associated with the administration of several anticancer drugs, including cisplatin. This active form of cell death is characterized by morphological changes such as cell shrinkage, chromatin condensation and fragmentation of DNA, which often requires the synthesis of new proteins called caspases<sup>(22, 23)</sup>.

Excretory ducts were dilated having wide lumen, degenerated epithelial lining and stagnant secretion. Dilatation of the ducts and stagnant secretion could be attributed to glandular damage and dysfunction. This finding was detected in the liver as cisplatin produced damaged structural integrity of the liver and hepatotoxicity<sup>(24)</sup>.

The connective tissue stroma presented thickening of the collagen bundles and heavy inflammatory cells infiltration. There were numerous lymphocytes, and mast cells. This result coincides with previous study on the kidney. Cisplatin produced chronic interstitial nephritis with interstitial fibrosis in humans<sup>(25)</sup>. In experimental animals, cisplatin induced renal interstitial fibrosis<sup>(26)</sup> that thought to cause irreversible renal dysfunction<sup>(27)</sup>.

L-Carnitine is a quaternary amine that is fundamental in skeletal muscle metabolism, in that it promotes fatty acids oxidation<sup>(28)</sup>. However, L-carnitine possesses some more complex activities in regulating gene expression and activity of caspases, the activation of which represents the compulsory

step for cell death execution<sup>(29, 30)</sup>. Additionally, other studies suggested that L-Carnitine had a direct or indirect antioxidant activity<sup>(31)</sup>. Histological examination of the parotid glands of L-Carnitine treated group revealed normal secretory and glandular elements with thin fibrous connective tissue septa. These findings are related to L-Carnitine treatment prior to cisplatin injection as experimental studies suggested that L-Carnitine is an antioxidant, anti inflammatory and cytoprotective agent used in the protection against cancer treatment-related normal tissue injury<sup>(32, 33)</sup>. It was also found that, antioxidants inhibit interstitial fibrosis induced by cisplatin<sup>(34)</sup>.

Apoptosis is energy dependent, natural, genetically-controlled process by which an organism eliminates unnecessary single cells<sup>(35)</sup>. The term "apoptosis" has been coined to explain the morphological processes principal to controlled cellular self-destruction. Proapoptotic Bax, another member of the Bcl-2 family, induces mitochondrial permeability transition and activation of downstream effector caspases, such as caspases 3, 6, and 7 that promote cell death<sup>(36)</sup>. Immunohistochemical examination of L-Carnitine treated group revealed no significant difference in Bax immunoreactivity compared with control group. While injection of cisplatin produced a significant increase in Bax immunoreactivity indicating increased apoptotic changes. These findings were in agreement with previous study on the inner ear. Bax protein expression (a proapoptotic protein) was clearly increased by cisplatin treatment and over expression of Bax promotes cell death<sup>(37)</sup>.

L-Carnitine treated group also revealed no detection of inflammatory cells. This was in agreement with previous study on the kidney. As acetylcarnitine was found to improve the histopathological structure as well as the kidney functions in case of cisplatin induced nephrotoxicity. Moreover, acetylcarnitine was reported to have antioxidative, antiapoptotic and anti-inflammatory properties that protect the tissues from cisplatin induced toxicity<sup>(38)</sup>.

In conclusion, cisplatin produced severe degenerative and apoptotic changes in parotid salivary glands. Acetyl L-Carnitine produced protection against cisplatin induced cytotoxicity as well as anti-apoptotic effect. Taken together, these findings suggest that Acetyl L-Carnitine is a supplemental agent to be usefully applied to reduce normal tissue damage by treatment of anticancer drugs such as cisplatin.

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