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## Histological and Immunohistochemical studies on the submandibular salivary glands of male albino rats after hexavalent chromium and vitamin C exposure.

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### ABSTRACT

A major development was the discovery that steel could be made highly resistant to corrosion and discoloration by adding metallic chromium to form stainless steel. This application along with chrome plating (electroplating with chromium) currently comprise 85% of the commercial use for the element. Trivalent chromium (Cr (III)) ion is possibly required in trace amount for sugar and lipid metabolism, although the issue remains in debate. In larger amounts and indifferent forms, chromium can be toxic and carcinogenic. The most prominent example of toxic chromium is hexavalent chromium (Cr VI). The present work was designed to evaluate the ameliorating role of vitamin C against the toxic and the possible carcinogenic effects of hexavalent chromium compound administered to male albino rats via drinking water for 3 months. Also to provide baseline information on the possible histopathological alterations developed in submandibular salivary glands. In this study 18 male albino rats (200-220gm body weight) were utilized. They were divided into three groups, 6 animals each. The first group was considered control group and received drinking tap water for 3months. The second group was the experimental group and received drinking tap water containing 300ug/L of Cr (VI) as chromium trioxide for 3months. The third group, received drinking tap water containing 300ug/L of Cr (VI) as group II. In addition the animals of group III were injected intramuscularly by 10mg/kg body weight of vitamin C, twice weekly for the whole duration of the experiment. Samples from the submandibular salivary glands were fixed in 10% buffered neutral formalin and prepared routinely for paraffin sectioning and staining for histological and immune histochemically immune expression of proliferating cell nuclear antigen (PCNA). Group II animals revealed marked histopathological degenerative changes in submandibular salivary glands. The striated duct appeared dilated, while granular duct showed stagnant eosinophilic secretion. The nuclei of the acinar cells showed hyperchromatic and abnormal mitosis. The acinar cells presented granular cytoplasm, while others showed empty vacuoles. The connective tissues stroma revealed increase in thickness. There was prominent inter-acinar edema. The blood vessels were congested and engorged with blood. The immune expression of PCNA in the nuclei of the acinar cells was intense after 3months daily administration of drinking tap water containing 300ug/L of Cr (VI). Group III received vitamin C in addition to Cr (VI), revealed some recovery to the normal structure of the submandibular salivary glands, and moderate immune reactivity to PCNA, which might revealed the protective antioxidant effect of vitamin C against cytotoxic effect of hexavalent chromium. The present study proved that the degenerative changes induced by hexavalent chromium in the submandibular salivary glands of male rats decreased in the group given vitamin C. The antioxidant effect of vitamin C was partly prevent the toxicity of hexavalent chromium.

**Keywords:** Submandibular salivary gland, Hexavalent chromium, PCNA.

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## INTRODUCTION

Pollution sources have become largely increased in last years and reached our life through water, air and food. Chromium (Cr) is one of the commonly used heavy metals and introduced into a variety of environmental media including water, soils and foods.

The hexavalent chromium is a human carcinogen (1). Hexavalent chromium compounds are potent toxic and carcinogenic agents, Cr (VI) is easily taken up by cells and is subsequently reduced to Cr (III). The formation of Cr (III) or other compounds such as Cr (IV) and Cr (V) might play a role in the adverse biological effects of hexavalent chromium (2).

Chromium compounds are found in the environment, due to erosion of chromium containing rock sand can be distributed by volcanic eruption. The concentration range in soils between 1 and 300mg/Kg, in sea water 5 to 800ug/L and in river sand lakes 26 ug/L to 5.2 mg/L (3). The acute oral toxicity for chromium (VI) range between 50 and 150 ug/L.

In the body, chromium (VI) is reduced by several mechanisms to chromium (III) already in the blood before it enters the cells. The chromium (III) excreted from the body, where as the chromate ion is transferred into the cell by a transport mechanism. The acute toxicity of chromium (VI) is due to its strong oxidational properties. The intracellular reduction of Cr (VI) to Cr (III) induces overproduction of reactive oxygen species which induced toxicity (4). After it reaches the blood stream, it damages the kidneys, the liver and blood cells through oxidation reactions. Hemolysis, renal and liver failure are the results of these damages (5). Chromium salts (chromates) are also cause of allergic reactions in some people. Chromates are often used to manufacture, amongst other things, leather products, paints, cement, mortar and anti-corrosives. Contact with products containing chromate can lead to allergic contact dermatitis (6).

Proliferating cell nuclear antigen (PCNA) was originally described in proliferating mammalian cells as a nuclear protein. The highly homologous nature of PCNA suggests that protein plays an essential role in DNA replication (7).

Proliferating cell nuclear antigen (PCNA) was involved in the cellular cycle (8) and could be identified in replicating cells of both benign and malignant lesions. Higher expression of this marker had been shown in aggregative tumors (9-11). Vitamin C as an antioxidant is one of the important water soluble vitamins and essential for collagen synthesis (12).

The endogenous and exogenous antioxidant are anti carcinogens (13). Free radicals and antioxidants were reported to be important in the protection against chromium toxicity (14).

## MATERIALS AND METHODS

The study was carried out on eighteen (18) adult male albino rats weighing about 200-220gm; they were caged in the animal room in the Faculty of Veterinary medicine, Cairo University throughout the experimental period (3months). The animals were maintained on stock diet and kept under fixed appropriate conditions of housing and handling. Animals in each group were caged in separate cages. The animals were classified into 3 groups (6 animals in each group) and treated as follow:

Group I (control group): received only drinking tap water for 3 months.

Group II (Experimental group): received drinking tap water containing 300ug/L of hexavalent chromium Cr (VI) as chromium trioxide (ADWIC, Laboratory chemicals, Egypt) for 3 months (The permissible concentration of Cr (VI) is 50-100ug/L- (Current drinking water standards) (15).

Group III (Experimental group): received drinking tap water containing 300ug/L of hexavalent chromium Cr (VI) as group II. In addition, the animals were injected by intramuscular injection of 10mg/kg body weight of vitamin C (Cevaryl 1000 mg/5 ml ampoule (Memphis Co. For Pharm. & Chemical Ind. Cairo, Egypt) two times per week for the whole duration of the experiment.

Samples from submandibular salivary glands were obtained after 3 months of the experimental period. Rats were sacrificed by cervical decapitation.

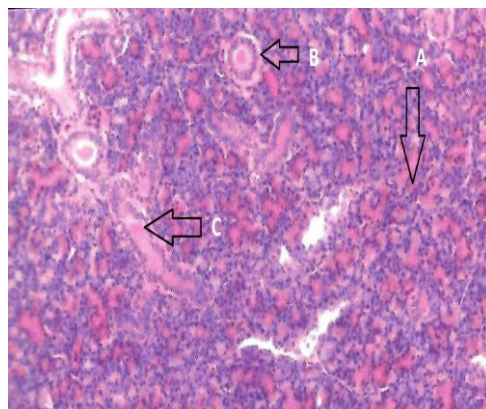
Specimen from submandibular salivary glands were washed, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin wax. Sections of 5-6 um in thickness were cutout, deparaffinized and stained with hematoxylin and eosin (H&E) and Mallory's trichrome stains for examination under the light microscope (16).

Immunohistochemistry for detection of PCNA was performed on paraffin sections and mounted on coated glass slides. Antigen was retrieved in citrate buffer (pH6.0) microwave digestion (2cycles of 12 minute each). Endogenous peroxidase was blocked with 0.05 % hydrogen peroxide for 30 min. After incubation with a 1:20 dilution of normal horse serum, the slides were incubated overnight at 4°C with primary antibodies (Dako, 1:50). Secondary antibodies associated with a streptavidin-biotin-peroxidase method were applied (DakoA/S). Diaminobenzidine was used as chromogen. All sections were counter-stained with hematoxylin. The sections were washed with phosphate buffered saline after each step. Negative controls were used using non-immune serum instead of the primary or secondary antibodies (17).

### RESULTS

Examination of control group sections revealed that the parenchyma of the submandibular salivary gland was formed of secretory end-pieces, excretory ducts and granular convoluted tubules. The secretory end-pieces or acini were found to be consisted of pyramidal shaped serous cells with round deeply basophilic nuclei in the basal half of the acinar cells. Few mucous acini and serous Demilune were present. The striated duct were formed of low columnar cells with centrally placed nuclei(Fig.1).The granular convoluted tubules were found to be larger than striated ducts and lined with high columnar cells with numerous eosinophilic granules in the supranuclear region and large ovoid deeply stained nuclei. Thick fibrous connective tissue was present between the lobes and lobules of the submandibular glands. Both acinar cells, striated ducts and granular convoluted tubules showed negative immune expression to PCNA (Fig.2).

The submandibular salivary glands of experimental groups that received daily dose of Cr (V1) revealed many histopathological and immunohistochemically alterations in the submandibular salivary glands. Hydropic degeneration of the cytoplasm. There were ill-distinct cell boundaries and vacuolization in the acini (Fig.3) The intercalated, striated and granular convoluted tubules were dilated and their cytoplasm showed complete signs of degeneration (Fig. 4).The granular ducts showed stagnant eosinophilic secretion. The nuclei of the acinar cells showed signs of pyknosis, hyper chromatics and abnormal mitosis (Fig.5). The interlobular blood vessels were dilated and engorged with blood (Fig. 6), also the inter-acinar blood capillaries were congested (Fig.7). Some acini were completely ruptured so that the nuclei were expelled out of the acini. There was a prominent inter acinar edema (Fig.8).There were prominent thick collagen fibers surrounding the acini (Fig.9) and around the intra and inter lobular ducts (Fig.10).



**Figure 1: Photo micrograph of submandibular salivary glands of albino rats of control group showing: a-normal acini b-striated duct c-granular duct (H&Ex40).**

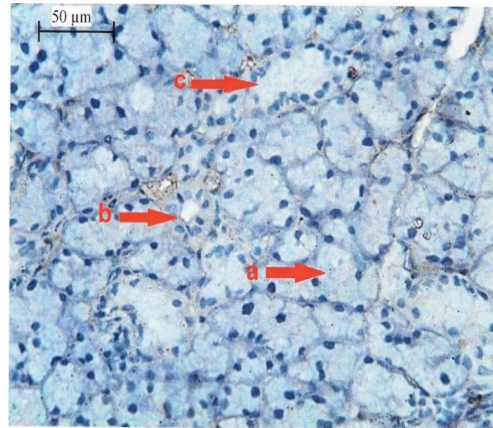


Figure 2: Photo micrograph of submandibular salivary glands of albino rats of control group showing negative immune reaction: a-acini b-striated duct c-granular duct (PCNAx100).

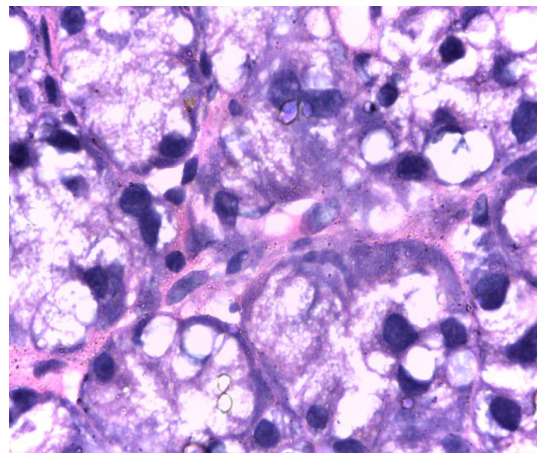


Figure 3: Photo micrograph of submandibular salivary glands of group II showing degenerative changes and vacuolization in the acini. (H&Ex1000).

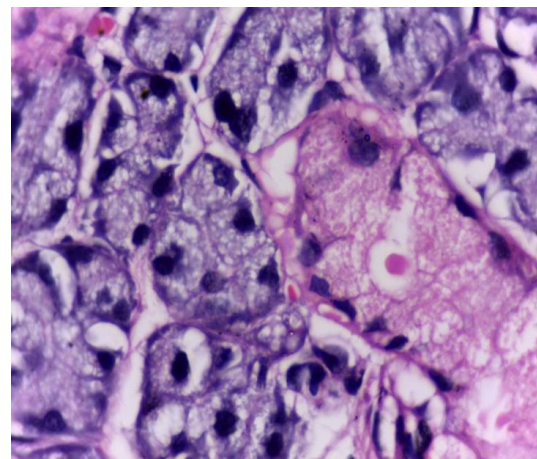


Figure 4: Photo micrograph of submandibular salivary glands of group II showing degenerative changes in striated duct and granular tubule. (H&E x400).



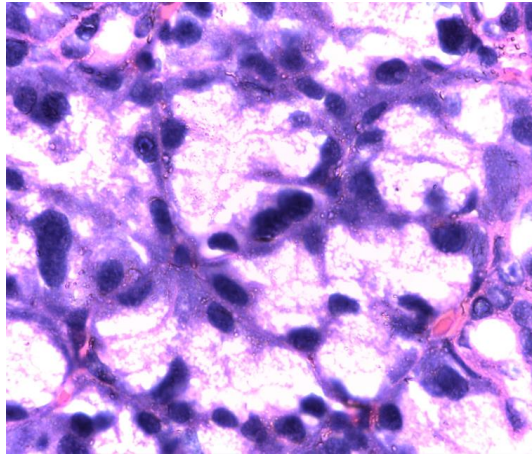


Figure 5: Photo micrograph of submandibular salivary glands of group II showing pyknosis, hyperchromatic and abnormal mitosis. (H&E x1000).

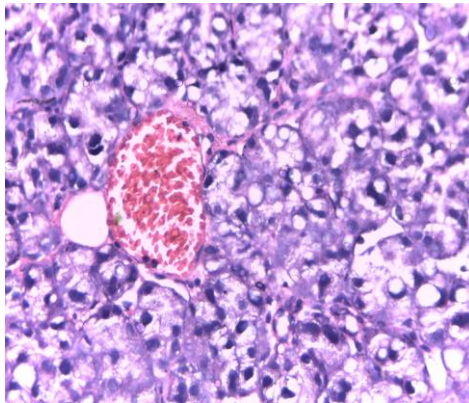


Figure 6: Photo micrograph of submandibular salivary glands of group II showing dilatation and congestion of blood vessels. (H&E x400).

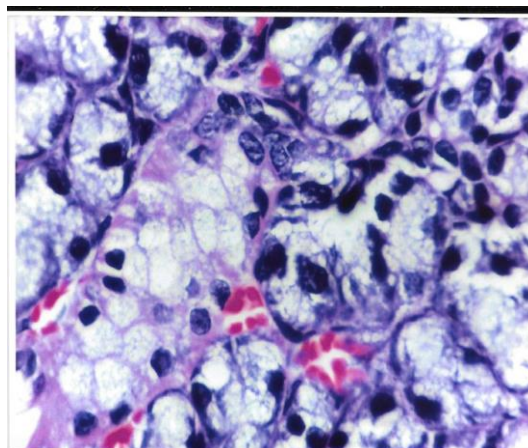


Figure 7: Photo micrograph of submandibular salivary glands of group II showing dilatation and congestion of inter-acinar blood capillaries.(H&E x400).

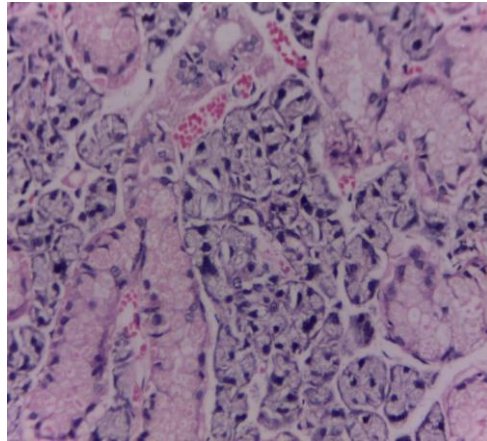


Figure 8: Photo micrograph of submandibular salivary glands of group II showing inter acinar edema. (H&E x400).

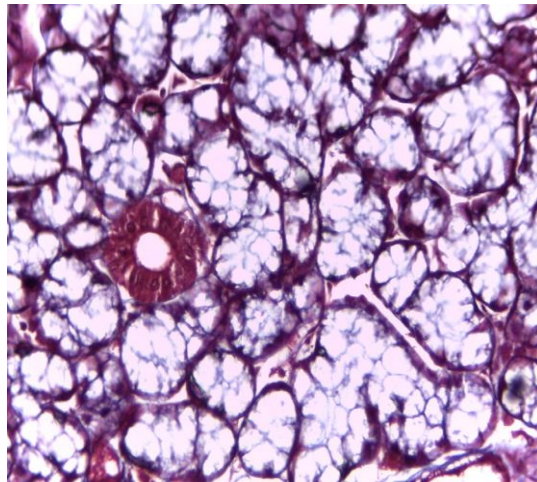


Figure 9: Photo micrograph of submandibular salivary glands of group II showing inter acinar thick collagen fibers. (Mallory's trichrome x400).

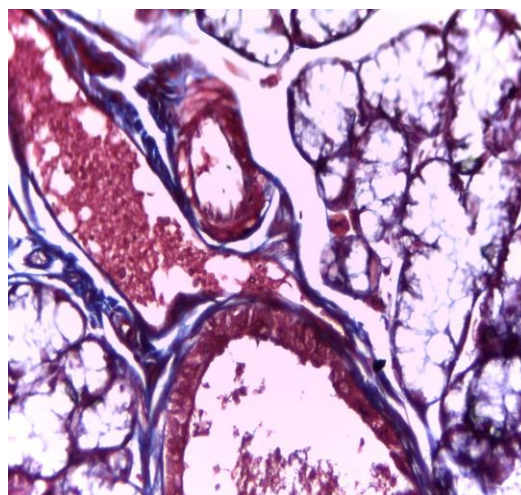
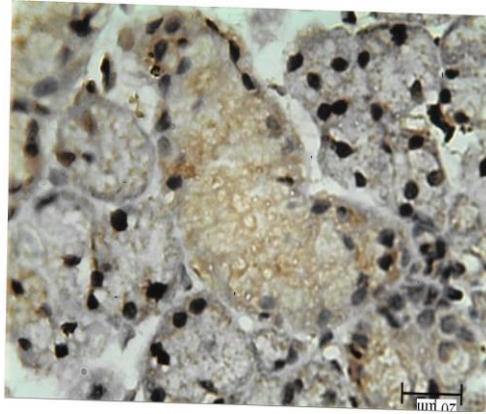
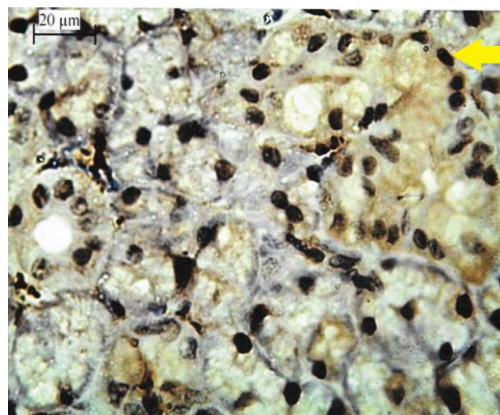


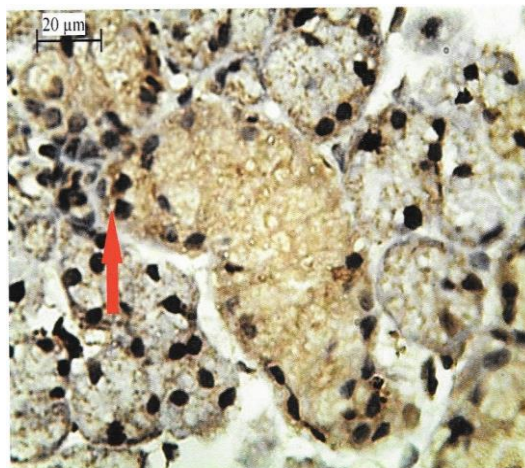
Figure 10: Photo micrograph of submandibular salivary glands of group II showing thick interlobular collagen fibers. (Mallory's trichrome x400).



**Figure 11:** Photo micrograph of submandibular salivary glands of group II showing intense immune expression in the nuclei of acinar cells and ductal epithelial cells. (PCNAx400).

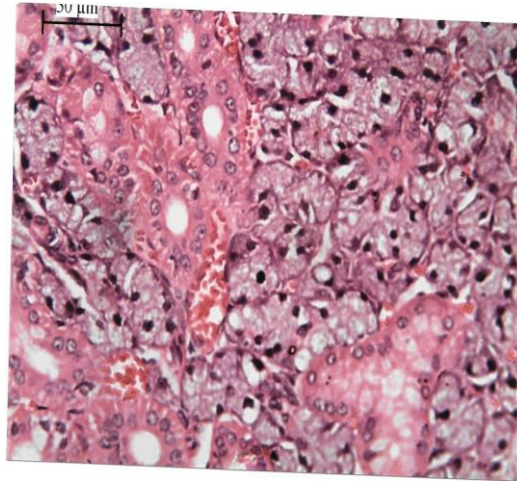


**Figure 12:** Photo micrograph of submandibular salivary glands of group II showing intense immune expression in the nuclei of striated ductal epithelial cells (arrow). (PCNAx630).

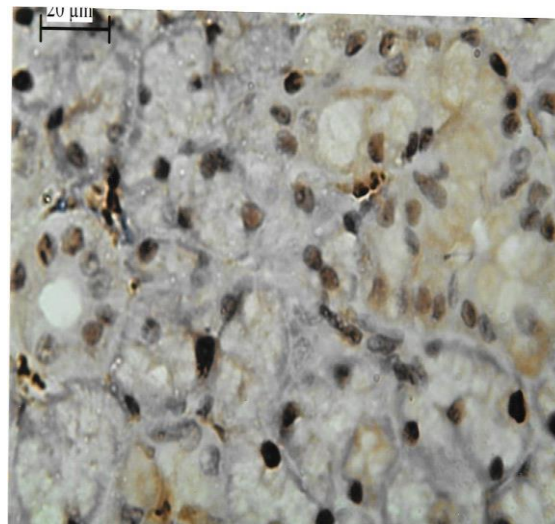


**Figure 13:** Photo micrograph of submandibular salivary glands of group II showing intense immune expression in the nuclei of granular convoluted tubule epithelial cells (arrow). (PCNAx630).





**Figure14: Photo micrograph of submandibular salivary glands of third group showing nearly normal structure but still blood vessels were congested. (H&E x400).**



**Figure 15: Photo micrograph of submandibular salivary glands of third group showing moderate immune expression in the nuclei of acinar cells. (PCNAx400).**

The nuclei of the acinar cells, nuclei of epithelial lining of striated and granular convoluted tubules of experimental group revealed intense immune expression to PCNA (Fig.11, 12, 13).The cytoplasm of the acinar cells showed weak positive immune reaction.

The submandibular salivary glands of group 3 that administered Cr (V1) and vitamin C showed some recovery to the normal structure but still some acini were distended and revealed signs of degeneration. The excretory ducts were dilated but lined with normal epithelium. Some blood vessels were dilated and congested (Fig.14).The nuclei of acinar cells, striated and granular convoluted tubules revealed moderate immune expression to PCNA (Fig.15).

#### DISCUSSION

The present investigation, showed that administration of chromium(Cr) to male rats via drinking water for 3 months revealed many histopathological and immunohistochemically alterations in the submandibular salivary glands of male albino rats. The acinar cells and ductal epithelial cells revealed degenerative changes and vacuolization of the cytoplasm. The connective tissue septa showed increased in thickness. The blood vessels were enlarged and congested. Inter-acinar edema was evident. Significant increase in micro nucleated polychromatic erythrocyte in bone marrow with increased chromium content in liver, kidney and plasma in



mice exposed to sub lethal dose of chromium (18). The effect of hexavalent chromium on the liver of rats and concluded that the toxic effect of chromium was due to the formation of highly reactive radicles and subsequent lipid peroxidation (19). The effect of chromium on the tongue of rats. They reported atrophy of the lingual papillae (20). They attributed this atrophy to the mechanism of epithelial reproduction through the cytotoxic effect of chromium. The cellular damage, morphological changes, chromatin condensation and DNA fragmentation might be due to the toxic effect of chromium (21). The effect of hexavalent chromium on the alimentary canal of F344 rats and B6C3F1 mice reported that, the histopathological findings in the rat's small intestine were generally similar to those reported for mice. In mice, cytoplasmic vacuolization was observed. Also villous atrophy and crypt cell hyperplasia were noticed. Vacuolization can be assigned as injury and thus suggests that damage to the villous epithelium resulted in crypt epithelial hyperplasia in mice (22). There are many potential causes of vacuolization including altered lipid metabolism, sequestration of absorbed material, autophagy, endoplasmic reticulum stress, and proteasome dysfunction (23, 24).

Inside the cell, indirect DNA damage may occur through the generation of oxygen radicals during intracellular reduction of Cr (VI) through the more reactive pentavalent and tetravalent chromium to Cr (III). Cr (III), the final product of intracellular reduction of Cr (VI), has been shown to interact directly with DNA and other macromolecules to induce chromosomal alterations and mutational changes (25, 26).

Hexavalent chromium reportedly induces reproductive toxicity and further inhibits male fertility in mammals. The molecular mechanism by which hexavalent chromium affects motility signaling in boar spermatozoa in vitro, indicated that Cr (VI) decreased sperm motility, protein phosphorylation, mitochondrial membrane potential and metabolic enzyme activity starting at 4  $\mu$ mol/mL following incubation for 1.5h (27).

Environmental and occupational exposure to chromium compounds, especially hexavalent chromium (Cr (VI)), is widely recognized as potentially hepatotoxic in humans and animals. Its toxicity is associated with overproduction of free radicals, which induces oxidative damage (28).

The proliferative activity of the submandibular salivary glands was studied in the present work using an immune histochemical staining of proliferating cell nuclear antigen (PCNA). The present study revealed intense expression of PCNA in the nuclei of the acinar cells after 3 months of Cr (VI) administration. This could be an indication for increasing proliferation rate, as an attempt to repair and renew the damaged cells. The accelerated proliferation might indicate an increased mutagenic risk on cells (29). PCNA which were involved in the cellular cycle could be identified in replicating cells of both benign and malignant lesions (8). On the other hand the reduction of Cr (VI) to the less permeable and bioavailable Cr (III) is thought to occur primarily in the stomach, as a mechanism of detoxification. Gastric reduction has been hypothesized to be efficient, such that oral exposure to Cr (VI) would not result in toxicity or carcinogenicity (30, 31).

Cr (VI) is genotoxic in a number of in vitro and in vivo test systems (32); however, the mechanisms of genotoxicity and carcinogenicity are not fully understood. Because Cr (VI) as chromate structurally resembles sulfate and phosphate, it can be taken up by all cells and organs throughout the body through non-specific transporters (33).

The present investigation proved that the degenerative changes induced by hexavalent chromium in the submandibular salivary glands of male albino rats decreased in the group given vitamin C. The antioxidant effect of vitamin C was evidently to partly prevent the toxicity of Cr (VI). The natural antioxidants strengthen the endogenous antioxidant defense (34).

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