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EVALUATION OF THE APOPTOTIC CHANGES INDUCED BY 5-FLUOROURACIL ON THE LINGUAL MUCOSA AND SALIVARY GLANDS OF MALE ALBINO RATS

(HISTOLOGICAL, HISTOMORPHOMETRIC AND IMMUNOHISTOCHEMICAL STUDY)

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ABSTRACT

Chemotherapy tends to stop the growth and eliminate cancer cells even at distant sites. Most of them lead to adverse side effects and induce changes in normal tissues. The aim of the present study was to evaluate of apoptotic changes induced by 5-fluorouracil on the lingual mucosa and salivary glands of male albino rats through clinical, histological, immunohistochemical and histomorphometric assessments. Twenty male albino rats were divided equally into two groups. Control group did not receive any type of treatment and study group received intraperitoneal injection of 5-FU (150 mg/kg) for 7 days. On the 14th day, the tongue and submandibular salivary gland specimens were dissected and processed. The study group showed histopathological changes as atrophy of the lingual papillae which confirmed statistically through histomorphometric analysis. The submandibular glands showed marked degeneration in the duct system and acini. The assessment of apoptosis was confirmed by immunohistochemical findings, there was highly statistically significant increase in the expression pattern of Bax and Caspase-3 with significant decrease in PCNA level in comparison to the control group. So, 5-FU caused deleterious histopathological and immunohistochemical alterations of the submandibular salivary glands and tongue mucosa.

KEYWORDS: Histomorphometry, 5-fluorouracil, Bax, Caspase-3, PCNA.

INTRODUCTION

Mucositis, an inflammatory condition, leads to damage of oro-esophageal mucosa following cytotoxic cancer therapies [1]. Chemotherapy is one of the cancer treatment regimens which used as the main or adjunctive treatment modality with the other types of treatments like surgery and radiotherapy [2]. It prevents the growth of cancer cells even at sites away from the origin of primary tumor [3]. Most chemotherapeutic agents lead to several adverse

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side effects on normal cell types and multiple organ systems that severely limit their activity [4]. These side effects include myelosuppression, nausea, vomiting, diarrhea, and stomatitis [5].

Fluorouracil (5-FU) is one of most common antineoplastic drugs that still included into chemotherapeutic strategies to treat solid cancers of the head and neck [6]. Block of DNA synthesis and replication is performed through thymidylate synthase enzyme inhibition. Interruption of this enzyme effects on DNA replication [7.8]. 5- FU, a pyrimidine analogue, is turned into the cell to different cytotoxic metabolites that induce cell cycle arrest. Several studies have shown that 5-FU has the potential to induce toxicity in various tissues [9], genotoxic as indicated by chromosomal damage [10], gastrointestinal dysfunction and enteric neurotoxicity [11].

Apoptosis is an ordered and orchestrated cellular process that occurs in physiological and pathological conditions. Cancer is one of the most common scenarios where apoptosis is stopped, leading to malignant cells survival [12]. Bcl-2 Associated X protein (**Bax),** a member of the Bcl-2 family, is a major effector of apoptosis [13]. Activation of BAX protein leads to increase permeabilization of mitochondrial outer membrane, release of cytochrome c, and activation of caspases [14]. Drugs that activate BAX hold promise as anticancer treatments by inducing apoptosis in cancer cells [15]. Cysteine-dependent aspartate specific proteases **(Caspases)** are a family of enzymes important to execute apoptosis. cellular signals and proteolytic activation of the caspases trigger the initiation of apoptosis cascades [16]. Upon the initiation of apoptosis, cleaved caspase-3 protein propagates an apoptotic signal through enzymatic activity, including poly ADP ribose polymerase (PARP) [17]. Proliferating cell nuclear antigen **(PCNA)**, a bridging molecule, plays an important role in cell growth and proliferation. Overexpression of PCNA promoted cell proliferation during DNA synthesis, tumorigenesis and inhibited cell apoptosis [18].

In this way, this experimental study was aimed to evaluate possible alterations in the tongue and salivary glands' mucosa which induced by fluorouracil through clinical, histological, histomorphometrical and immunohistochemical assessment for apoptotic changes in albino rats.

MATERIALS AND METHODS

Materials:

A stock solution of 5-FU 150 mg was obtained from ACDIMA International (AiT) Shanghai Xudong Haipu Pharmaceutical Co., Ltd.

Experimental design:

Ethical approval:

The present study was approved by the research ethical committee (REC), Faculty of dentistry, Suez Canal University, approval No. (252/2019), and followed the guidelines set by the committee for the experimental studies.

Sample size calculation:

It was performed using G*Power version 3.1.9.2**,** University Kiel, Germany [19]. Copyright (c) 1992-2014. The effect size d was 1.6 using alpha (α) level of 0.05 and Beta (β) level of 0.05, i.e., power = 95%; the estimated sample size (n) was a total of 20 samples.

Animal's grouping:

Twenty male albino rats weighing 260.8 ± 11.7 gm were caged in the animal house of Faculty of Dentistry, Suez Canal University, Ismailia. They were supplied with standard natural diet and tap water *ad libitum* through the whole experimental period (14 days). They were maintained under good ventilation.

The animals were divided into two equal groups, as followed: **Control group (n=10)** did not receive any type of treatment. **Study group (n=10)** received

intraperitoneal injection of 5-FU (150 mg/kg) for successive 7 days. On the 14th day after treatments, the rats were euthanized by cervical dislocation. All the rats were weighed at the beginning and the end of the experiment and the weights were recorded.

Histopathological analysis:

Tongue and submandibular salivary glands were dissected out, fixed in 10% neutral buffered formalin solution for 24 hours, then tissues processed, embedded in paraffin, sectioned into 5µm, mounted, and stained with:

- Hematoxylin and eosin for light microscopic examination to evaluate the possible histopathological changes.
- Masson Trichrome was used for detection of collagen fibers. The slides were examined and photographed by E-330 Olympus digital camera.

Immunohistochemical (IHC) processing and analysis:

Five µm sections were cut and mounted on positively charged slides. The immunostaining was performed using rabbit polycolonal mouse antibody to **Bax** (Santa Cruz Biotechnology, Cat. No. sc-526), rabbit polycolonal antibody to **Caspase 3** (R&D Systems, Cat. No. AF-835) and mouse anti-rat antibody to **PCNA** (Santa Cruz Biotechnology, Cat. No. sc-56). The steps of IHC staining were followed according to manufacturer's instructions.

Digital Image analysis:

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

- Histomorphometric analysis of the mean total collagen density in the submandibular salivary glands and lingual mucosa
- Histomorphometric assessment of the mean length and width of the lingual papillae among the two groups.
- In immunohistochemical analysis through measuring the optical density of immunostained cells and digitizing the slides under 400X objective magnification in each group.

Statistical analysis:

All data were calculated, tabulated, and statistically analyzed using the computer program SPSS software for windows version 22.0 (Statistical Package for Social Science, Armonk, NY: IBM Corp) at significant levels 0.05 (P- Value ≤0.5). **A) Descriptive data** were calculated in the form of Mean ± Standard deviation (SD). **B) Independent Student's T-test** were performed for comparison of the mean differences between the two groups for each measurement.

RESULTS

Clinical findings:

There was loss of weight in all rats receiving 5-flurouracil (study group). The unpaired t-test revealed highly significant difference between the weights of the rats in the study group (184.6 ± 12.1) in comparison to control group (260.8 ± 11.7) (Table1).

Histopathological evaluation:

Control group:

A) **The submandibular glands** consisted of connective tissue capsule that surrounded the parenchyma and divided it into lobes and lobules. The parenchyma was formed of secretory end-pieces known as serous acini and ductal system. The acini were composed of narrow lumen and lined by pyramidal cells with pale granular cytoplasm and basal large, rounded nuclei. The acini were more abundant as compared to the salivary ducts. The ductal system consisted of intercalated, granular convoluted tubules (had large number of secretary granules), striated, and excretory ducts (**figure 1-A**).

B) The tongue: its dorsal surface covered by keratinized stratified squamous epithelium and connective tissue layer within the underlying skeletal muscle fibers. The skeletal muscles were formed of interlacing bundles running in different directions. It was characterized by numerous slender filiform papillae with sharp conical projections and lined by smooth keratinized stratified squamous epithelium (**figure 1- E**). Few fungiform papillae were between the filiform papillae. At the summit of each fungiform, there was a peripherally located single taste bud papilla (**figure 1- F**). There were no lingual papillae in the lingual ventral surface.

Study group:

A) The submandibular glands showed significant degree of degenerative changes of their acini in addition to marked pathological changes in their ductal epithelium. Some acini showed cytoplasmic vacuolization **(figure 1-B)**, some acini lost its normal appearance **(figure 1- D)**, while the other appeared normal. Striated ducts appeared shrunken and surrounded by empty space **(figure 1-C)**. Most of GCTs showed signs of degeneration in the form of loss of granules, ill-defined outlines, cytoplasmic vacuolization and clumping of the cytoplasm **(figure 1- D)**. The epithelial lining of excretory ducts appeared atrophic with loss of their pseudo-stratification. Loss of normal architecture of the secretory portion. Stagnation of secretory material in some dilated ducts. Shrunken ducts appeared as hollow space around striated duct. Large, dilated blood vessel engorged with red blood cells **(figure 2- D)**.

B) The tongue: Obliteration of taste buds of fungiform papillae was observed (**figure 1-G**). Filiform papillae showed shortening or flattening, with few shallow epithelial ridges. Some areas showed focal loss of some filiform papillae (**figure 1-H**). Features of apoptotic cells get clear in the lingual epithelium in the form of crescentic or irregular nucleus in addition to nuclear condensation or pyknotic nuclei (**figure 1-I**). There was vacuolation of some epithelial cells, areas of hyperkeratosis in addition to focal separation of the keratin layer from underlying epithelial cells. Lamina propria showed dilated congested blood vessels with mild infiltration of mononuclear cells. Moreover, the underlying skeletal muscle fibers were widely separated (**figure 1-J**). Lingual Von Ebner salivary glands showed a lot of vacuolization (**figure 1-K**). The ventral tongue surface thinning of epithelial lining (**figure 1-L**).

Masson Trichrome evaluation:

It was used for assessment of the density of type I collagen. Collagen fibers appeared in blue or green color. In the study group, collagen fibers in the lamina propria of submandibular salivary glands and lingual mucosa were looser and disorganized in comparison with control group as an indication for marked degeneration (**figure 2, A-D**).

Histomorphometric analysis of the mean total collagen density in the submandibular salivary glands and lingual mucosa showed highly statistically significant decrease in the study group (67.7 ± 6.7 and 32.2 ± 8.4) respectively in comparison to control group (114.7 ± 8.2 and 74.3 ± 13.3) respectively (**table 1**).

Morphometric analysis:

It was helpful in detecting the mean length and width of filiform lingual papillae. It revealed a highly statistically significant decrease in the study group (771.0 ± 64.2 and 165.2 ± 17.8) in comparison to control group (1482.1 ± 116.9 and 328.6 ± 36.4) **(Table 1)**.

Immunohistochemical (IHC) evaluation:

Immunohistochemical expression of PCNA was helpful in assessment of the cell proliferation. In control group I, it appeared as intense brown nuclear staining reaction which localized in the basal layer

of the submandibular salivary glands and extended to parabasal cell layers of the lingual epithelium (**figure 2-E, F**) while in the study group, PCNA expression decreased (**figure 2-G, H**). Statistical analysis of PCNA expression in the submandibular salivary glands and lingual mucosa showed highly statistically significant decrease in group II (47.1 ± 13.7 and 12.5 ± 2.9) respectively in comparison to group I (170.4 ± 45.5 and 38.3 ± 3.6) respectively (**table 1**).

Assessment of Apoptosis was done through evaluation of Bax and Caspase 3 immunostaining in the examined tissues of the two groups. In control group, Bax appeared as mild cytoplasmic reaction in the ductal epithelium of the submandibular salivary glands and restricted to the superficial layers of the lingual epithelium (**figure 2-I, J**). In study group, Bax expression increased and appeared among salivary glands acini, ducts and extended to all over lingual epithelial thickness (**figure 2-K, L**). There was highly statistically significant increase in group II (152.7 ± 12.1and 121.8 ± 17.5) respectively in comparison to group I (33.2 ± 13.5 and 26.3 ± 3.9) respectively (**table 1**).

As for Caspase-3 in control group, its expression showed mild cytoplasmic reaction in the ductal, acinar, and lingual epithelium (**figure 2- M,N**). While in the study group, there was moderate to strong positive cytoplasmic and nuclear immunoreactions of ductal cells in addition to the lingual epithelium (**figure 2- O, P**). There was highly statistically significant increase in group II (133.1 ± 9.7 and 109.2 ± 9.3) respectively in comparison to group I (34.0 ± 4.9 and 66.7 ± 16.9) respectively (**table 1**).

TABLE (1) Variables Statistical analysis using Student's T-test.

Variables	Group I	Group II	Significance
Animal weight	$260.8^{a} \pm 11.7$	$184.6^{b} \pm 12.1$	0.00**
Collagen density (SG)	$114.7^{a} \pm 8.2$	$67.7^{b} \pm 6.7$	0.00**
Collagen density (LM)	$74.3^{a} \pm 13.3$	$32.2^{b} \pm 8.4$	0.00**
Papillae length	$1482.1^{a} \pm 116.9$	$771.0^{b} \pm 64.2$	0.00**
Papillae width	$328.6^{a} \pm 36.4$	$165.2^{b} \pm 17.8$	0.00**
PCNA optical density (SG)	$170.4^{a} \pm 45.5$	$47.1^{b} \pm 13.7$	0.00**
PCNA optical density (LM)	$38.3^{a} \pm 3.6$	$12.5^{b} \pm 2.9$	0.00**
Bax optical density (SG)	$33.2^{b} \pm 13.5$	$152.7^{a} \pm 12.1$	0.00**
Bax optical density (LM)	$26.3^{b} \pm 3.9$	$121.8^{a} \pm 17.5$	0.00**
Caspase optical density (SG)	$34.0^{b} \pm 4.9$	$133.1^{a} \pm 9.7$	0.00**
Caspase optical density (LM)	$66.7^{b} \pm 16.9$	$109.2^{a} \pm 9.3$	0.00**

*****,a,b; mean significant difference between groups using independent T-test at P value ≤ 0.05***

SG: submandibular salivary glands

LM: lingual mucosa

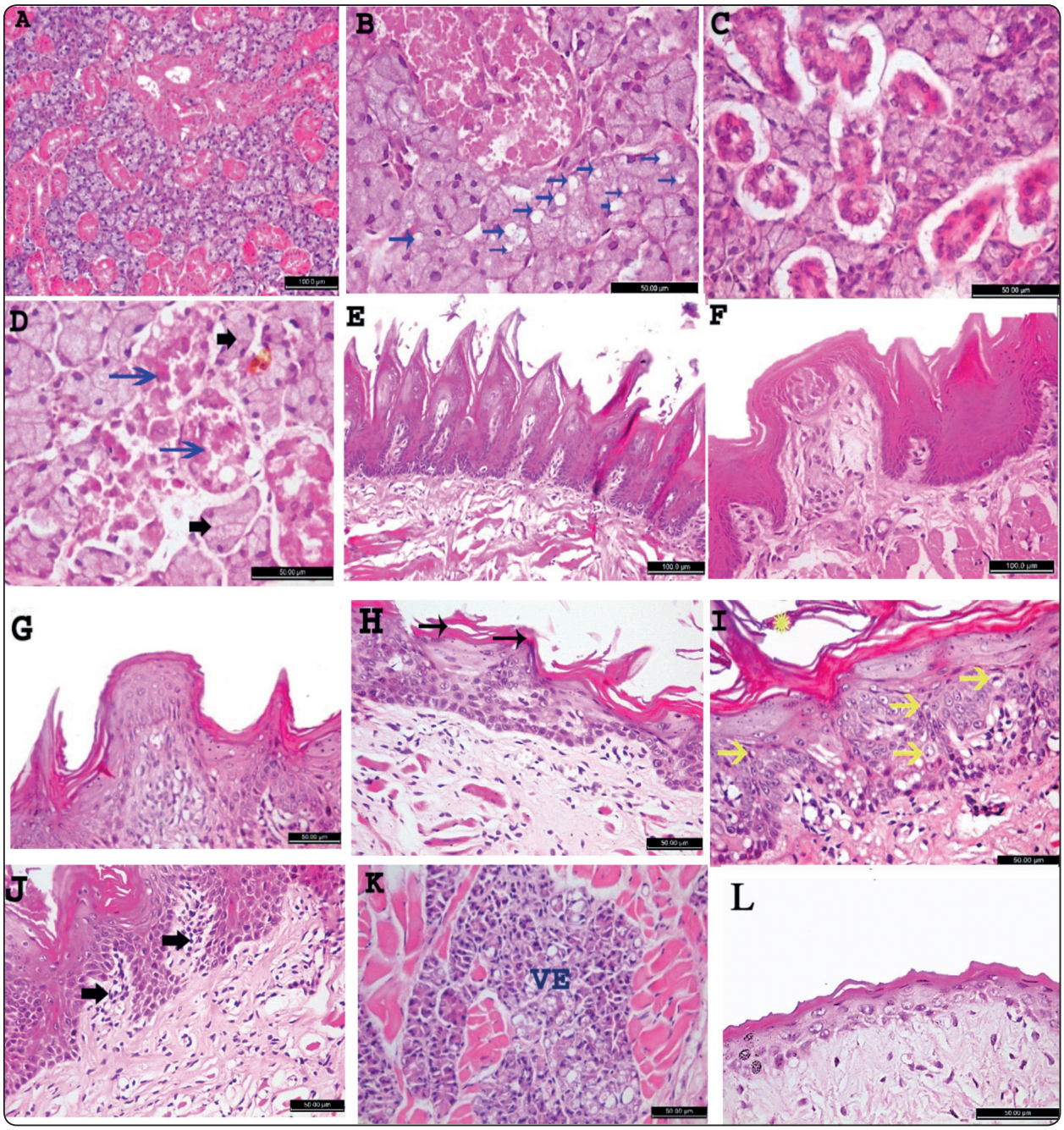

Fig. (1): Photomicrographs of H&E section showing: A): Submandibular salivary glands of control group with normal architecture of the acini and the ducts. B): Submandibular salivary glands of FLU treated group showed vacuolated acini (blue arrows). C): Shrunken striated ducts. D): GCT showing ill-defined border, vacuolated cells (blue arrows) and loss of granulation, some acini showing loss of normal architecture (black arrows). E): Dorsal surface of the control group showing filiform papillae. F): Fungi form papillae with taste bud on the superior part. G): Dorsal surface of the tongue of FLU treated group showing atrophied fungiform with obliteration of taste buds. H): Deformed filiform papillae, with areas of focal loss (arrows). I): Atrophic filiform papillae with separation of keratin layer (*), the epithelium showed apoptotic cell figures (yellow arrows). J) Inflammatory cell infiltrate in the lamina propria beneath the papillae, [arrows]. K) Von Ebner salivary glands (VE) showing a lot of cytoplasmic vacuolization. L) Atrophy of the epithelium lining of the ventral surface.

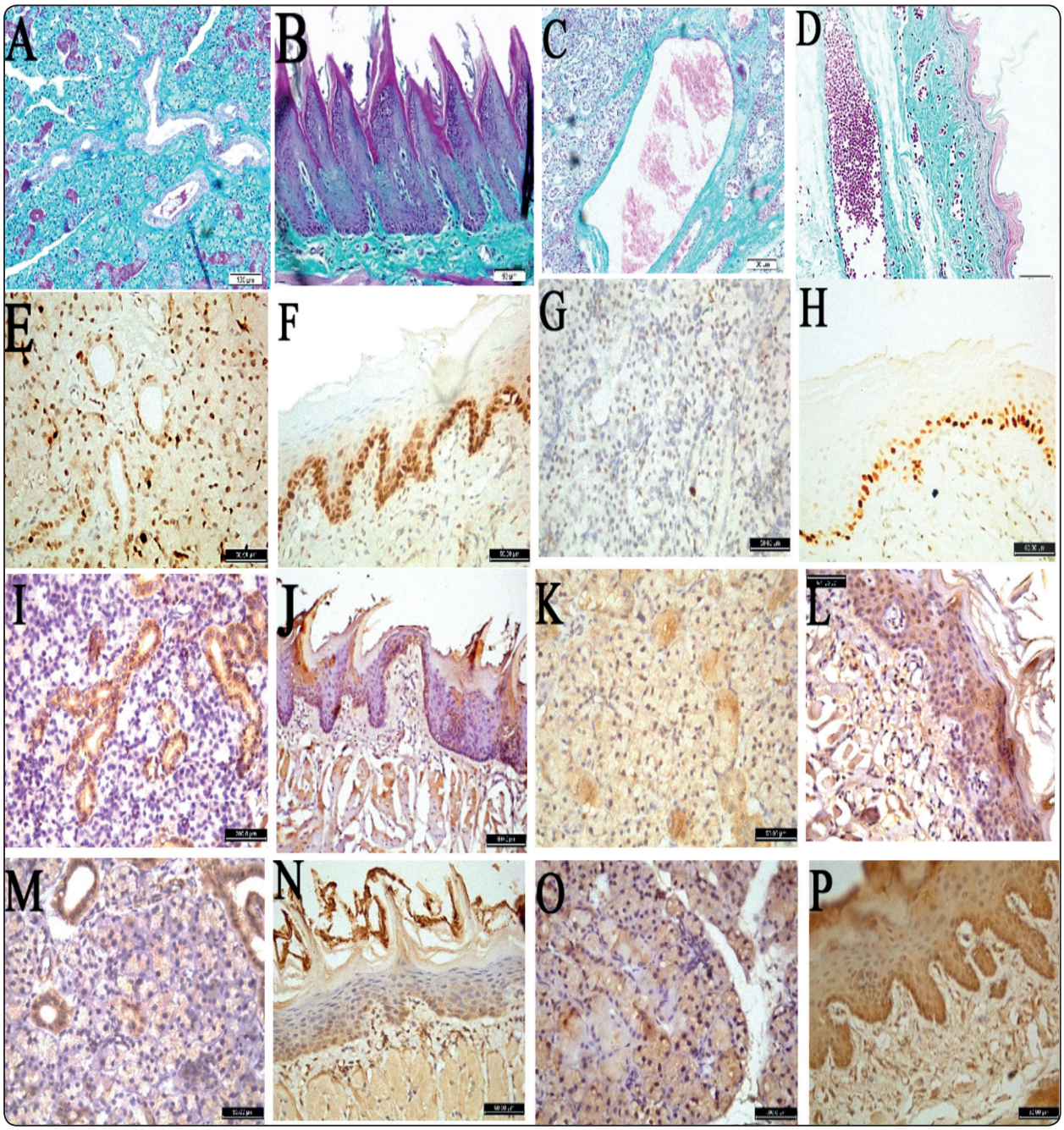

Fig. (2) Masson Trichrome sections showing: A, B): Control group with normal appearance and distribution of collagen fibers. C, D) Study group showing looser and disorganized collagen fibers. Immunohistochemical sections showing E, F): in control group, intense PCNA nuclear positive immunoreaction in both acinar and ductal epithelial cells and limited to the basal and parabasal layer of lingual mucosa. G, H): PCNA immunoreaction decreased in the study group. I, J): Bax immunoreaction showing mild cytoplasmic immunoreaction in the control group. K, L): The expression increased in the study group. M, N): Caspase 3 immunoreaction showing mild cytoplasmic immunoreaction in the control group. O, P) The expression gets increased in the study group.

DISCUSSION

Modern anticancer agents face many challenges to the oral mucosa integrity. The major problem is lack of selectivity. So, these agents restrict the oral epithelium proliferative nature that led to thin or ulcerated epithelium [20]. Therefore, this study was performed to evaluate the apoptotic changes induced by 5-fluorouracil on the lingual mucosa and salivary glands of male albino rats histologically and immunohistochemically.

In this research, administration of 5-FU through intraperitoneal route was used because it is considered more effective and less toxic to rats [21]. There was a highly statistically significant loss of weight in the study group which may be due to diarrhea or conditioned aversions to food which developed by chemotherapy [22].

The histological findings in this research were in accordance with other researchers that reported similar changes by other cytotoxic drugs as irinotecan [23] and methotrexate [24]. The changes occurred for rat's subjected to chemotherapy might be due to disruption of protein synthesis and increase of apoptosis [25]. The epithelial atrophy and collagen degeneration might be associated with the inhibitory effects of chemotherapeutic agents on DNA replication which impaired the renewal capacity, and inhibition of mitosis [26]. The free radicals might play a role in the intracellular damage during the chemotherapy metabolism. These radicals increase vascular permeability which enhance cytotoxic drug uptake to oral mucosa [27].

The filiform papillae are considered as a mirror which reflect the general health because they have high metabolic activity. So, any drug toxicity may result in atrophy of these papillae [28]. Numerous congested blood capillaries might be related to the release of pro-inflammatory cytokines as tumor necrosis factor, interleukin-1B, and prostaglandins which might cause damage through increasing vascular permeability and enhancing the cytotoxic drug uptake to the oral mucosa [28]. Retaining the secretion in some ducts might be due to defect occurred on the myoepithelial cells that led to glandular dysfunction. Furthermore, the accumulation of the secretory granules led to degenerative changes due to its content of proteolytic enzymes. These cells were replaced by connective tissue [29].

Assumed that apoptosis plays an important role in the mechanism of chemotherapy- induced regression. Therefore, the rate of proteins that regulate apoptosis may be predictive to chemotherapy responses [30]. A recent study documented that the oral mucosa treated with chemotherapy showed signs of apoptosis as nuclear condensation, pyknosis, chromatin margination and cytoplasmic vacuolation [23]. This could be related to DNA damage and inhibition of DNA replication step of topoisomerase I enzyme which responsible for cell division [31].

Our study showed that group II had a significant increase in the expression of Caspase-3 and Bax with decrease in the expression of PCNA. This indicated that 5-FU significantly induced apoptosis and decreased surviving when compared with the control group. On line with our study, Ozel et al., (2010)) [29] documented that there was no cellular proliferation in the acinar cells of the rabbit's submandibular glands with PCNA in the 5-FU-treated group and higher rate of apoptosis according to TdT-mediated dUTP nick end labeling (TUNEL. Also, Zahawi et al., (2017) [32] reported that administration of 5-FU was accompanied with a significant increase in caspase -3 and a reduction in Ki-67 expression. In other study, increasing the expression of Caspases-3 and 7 was remarkable in 5-FU-treated colon cancer cells compared to negative control [33]. Consistent with our findings, there was significant increase in Bax expression in fluorouracil-induced injury [34.35]. In different kinds of cancer, Bax overexpression was documented to be strongly associated with chemotherapeutic effects [36.37]. Silencing of Bax was contributed to 5-FU resistance and increasing the Bax expression enhanced the 5-FU susceptibility [38]. The upregulation of the Bax/Bcl-2 ratio led to increase

of outer mitochondrial membrane permeabilization and release of mitochondrial proteins such as cytochrome c [39]. Disruption in mitochondria membrane potential which induced caspases activation would be led to increase the apoptosis [40].

CONCLUSIONS

5-fluorouracil has a deleterious effect on the mucosa of tongue and submandibular salivary gland leading to marked histological and immunohistochemical changes. All these findings highlight the critical role of these markers in regulating the cellular response to 5-FU. So, the patients receiving 5-FU should be managed to avoid the risk of oral complications.

RECOMMENDATIONS

Patients receiving chemotherapy who have oral mucositis should require supportive care measures such as use of maintenance of good oral hygiene, pain management, liquid diet supplement, prophylaxis against infections and parenteral nutrition. Further studies will be needed to investigate the ability of anti-inflammatory phytochemicals to decrease the cytotoxic effects of chemotherapy.

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