

Effects of Kurdistan Honey on the Tongue of Chemotherapy Treated Albino Rats (Immunohistochemical Study)

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Rec date: Jul 16, 2014, Acc date: Aug 21, 2014, Pub date: Aug 23, 2014

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Abstract

Background and Objectives: Mucositis can be a dose-limiting toxicity of cancer chemotherapy with direct effects on patient survival; therefore an effective intervention is considered a high priority in cancer patient. The aim of the present study was to evaluate the effectiveness of honey as a preventive treatment for the methotrexate (MTX) induced oral mucositis.

Materials and Methods: In current study forty females Albino rats, weighing 250-300 g were used in the study. For the induction of oral mucositis, 60 mg/kg of MTX was administered intraperitoneally to each animal in the study group at day 4. The control animals were intraperitoneally injected by normal saline in the same manner and dose like MTX.

At the beginning of the experiment, the rats in each group were randomly divided into two groups: Distilled water treated group and honey treated group (10 animals each). A volume of distilled water equal to honey was given by intragastric gavage tube, while the other group was gavaged with honey at a dose of 2.5 g/kg two times daily (with a total of 5 g/kg/day). The animals were sacrificed at day 8. In each experiment, the middle third of tongue was removed for histopathological and immunohistochemical analysis using Ki-67 and Bcl-2 immunolabeling.

Results: The result showed that, the MTX/honey group showed a significant increase in the thickness of the epithelium ($p < 0.01$), significant decrease in the number of congested blood vessels in the connective tissue of rat tongue mucosa ($p < 0.01$), non significant increase in the Ki-67 immune expression ($p > 0.01$), and significant increase in Bcl-2 immune expression ($p < 0.01$) in comparison with the methotrexate/water group.

Conclusion: Natural honey at a concentration of (5 g/kg/day) produced protection against methotrexate induced tongue mucositis and therefore can be used as a protective natural product to oral mucosa against methotrexate induced cytotoxicity.

Keywords: Mucositis; Tongue; Honey; Methotrexate

Introduction

Cancer is a broad group of diseases involving unregulated cell growth. In cancer, cells divide and grow uncontrollably, forming malignant tumors, and invading nearby parts of the body and may also spread to more distant parts of the body. It is characterized by a series of cellular and genetic changes [1]. The treatment usually involves chemotherapy, radiotherapy, or surgery, either as a single therapy or in combination. Methotrexate (MTX) represents one of the most potent anti-tumor drugs, it was used at higher doses as a cancer therapy and it is used at much lower doses to treat rheumatic diseases and psoriasis [2]. Methotrexate is an antimetabolite inhibiting dihydrofolate reductase blocking the reduction of dihydrofolate to tetrahydrofolic acid. Depletion of tetrahydrofolic acid leads to a decreased thymidylate and purine biosynthesis, resulting in a decreased DNA synthesis. This effect on reducing DNA formation and cell turnover is responsible for both the therapeutic effect and the more common side effects [3].

Mucositis is the inflammation and ulceration of the mucosal membranes and it is one of the side effects of chemotherapy and radiotherapy. Unfortunately such therapy affects all rapidly dividing cells whether neoplastic or not. Consequently, the lining of the oral cavity, like the ventral tongue mucosa is at high risk of side effects [4].

Oral mucositis is an inflammatory, painful, and debilitating condition, occurs in 30 to 40% of patients receiving chemotherapy, 60% of patients receiving radiation therapy and 92% of patients receiving both chemotherapy and radiation therapy [5]. It is caused by a multi-step biological process, it is an inflammatory response of the oral mucosa that pathophysiology is complex and multifactorial. Histopathological evaluation of mucositis lesions shows mucosal thinning, caused by apoptosis and depletion of the epithelial basal layer, with subsequent denudation, its begin in the epithelium but then progress to involve the connective tissue [6].

The difficulty in eating and drinking was reported in nearly 90% of the examined patients with oral mucositis, and resultant weight loss in approximately 85% [7]. It can also lead to a reduction in total dose delivered to the tumor bed and unscheduled treatment breaks. This

can have a detrimental effect on local tumor control and thus patient survival [8].

Honey is the substance made when the nectar and sweet deposits from plants are gathered, modified and stored in the honeycomb by honey bees. Honey has antibacterial, anti-viral, anti-fungal and antioxidants properties, and can be used for treatment of nausea, cough, cold, and effective against cancer growth [9]. Topically applied honey was also used for treatment of radiation - induced mucositis in cancer patients [10].

Chemotherapy-induced oral mucositis is an important dose-limiting and costly side effect for which there is no definitive prophylaxis or treatment. The current study was aimed to evaluate the preventive role of honey (5 g/ kg /day) administered by gastric gavage for the MTX-induced tongue cytotoxicity. As variables to evaluate the grade of protection, we used histological and immunohistochemical investigations to clarify its effect on cell proliferation and cell apoptosis.

Materials and Methods

Rats and housing

In current study forty females Albino rats, weighing 250-300 g were supplied and cared in the Animal House of College of Medicine, Hawler Medical University, Erbil, Kurdistan Region of Iraq. The animals were kept under a standard laboratory conditions and maintained on a 12 hour light/dark cycle at $20 \pm 5^{\circ}\text{C}$, fed with a standard rat chow and allowed to drink water ad libitum. The research project was approved by the Research Ethics Committee at College of Dentistry, Hawler Medical University under protocol.

Preparation of honey

Natural unprocessed raw honey was obtained from Merkasour, Kurdistan region of Iraq. The initial solution of honey was freshly prepared by dissolving 50 g of this brown, thick, and sticky honey in 40 ml of distilled water (one ml contain 1.25 g honey). The mixture was filtered first with a fine muslin cloth and then with filter paper (Whatman no.1).

Induction of mucositis

Fijlstra et al. [11] found that the typical clinical signs of mucositis, such as a decreased food intake, weight loss, and diarrhea, were present in most methotrexate treated rats (60 mg/kg) from the second day until the fifth day of the experiment, after which rats started to recover. In the fourth day of the experiment, the histological and clinical symptoms of the induced mucositis were most severe. For this reason the animals were scarified four days after intraperitoneal injection of MTX. Al-Refai et al. (2014) found that doses of MTX less than this dose can cause oral mucositis [12].

Experimental design

The rats were randomly divided into two groups:

Control groups: Consist of distilled water treated group and honey treated group (10 animals each), the treatment continue for eight days. In the distilled water treated group, a volume of distilled water equal to honey was given with gavage tube, while the honey-treated group was gavaged with honey at a dose of 2.5 g/kg two times daily, with a total of

5 g/kg/day [13]. A physiological saline (0.9% NaCl) in a similar dose of MTX (60 mg/Kg) interrupt the treatment at day four and administered intraperitoneally instead of MTX.

Study or MTX- treated groups: Consist also of distilled water treated group and honey treated group (10 animals each), the treatment continue for eight days. They were gavaged by distilled water or by honey in a similar way like control group, but intraperitoneal injections of the MTX interrupted the treatment at day four.

Histopathological analysis

The animals were sacrificed by over dose of anesthesia at day 8 and the middle third of the tongue was removed for histopathological analysis. Samples were then fixed in neutral buffered 10% formalin, processed for H&E, and for immunohistochemical analysis using Bcl-2 and Ki-67 immunolabeling.

For histological analyses, the picture captured at 400x magnification, and three microscopic fields from each cheek mucosal epithelium section were registered between the external epithelial surface and the epithelial crista (major epithelial thickness) and performed in each of the three photograph fields. At every 10 measurements of epithelial thickness, one was repeated to assess intra-examiner reproducibility. All analysis was blind to the type of the sample. The presence of inflammatory infiltration was also evaluated at 400x magnification, and each microscopical field was labeled 0 (absence of inflammatory cells) or 1 (presence of inflammatory cells) respectively. For assessment of the number of blood vessels in each field, structures with endothelial lining and with red blood cells in their interior were counted at 20x magnification. At every 10 fields, a count was repeated to assess intra examiner reproducibility. All analysis was blind to the origin of the sample.

Immunohistochemical stains and analysis using Ki-67 and Bcl-2 immunolabeling

Cell proliferation was assessed by Ki-67 immunohistochemistry, while the anti apoptosis was assessed by Bcl-2 immunostaining and were performed using monoclonal Mouse Anti-Human Ki-67 Antigen, Clone MIB-1, Code No. M 7240 staining system, and a monoclonal Mouse Anti-Human Bcl-2 Oncoprotein Clone 124 Code No 1587 ready to use N-series primary antibody, for use with DakoEnVision TM , EnVision TM double staining and LASAB TM 2 systems. The staining procedure sections of the instructions included with each detection system were followed. Positive and negative controls were run simultaneously with biopsy specimen.

Positive cells expressing Ki-67 were identified by brown nuclei, while Bcl-2 was demonstrated brown cytoplasmic staining. To ensure the objectivity of the analysis, the evaluation was carried out by two independent observers. Five sections were randomly chosen for each animal. Approximately 1000 cells from cell population were counted by two observers at a magnification of 400x and the percentages of Ki-67 and Bcl-2 positive cells were calculated. All microscopic analyses were performed using a light microscope (Olympus, Tokyo, Japan).

The levels of Ki-67 and Bcl-2 expression were evaluated according to the scoring system of Seleit et al. [14]. The application of this system gives a score cells [(absent: <1%), (mild: 1-10%), (moderate: 10-50%), (strong: >50%)].

Statistical analysis

Statistical analysis was performed using Bonferroni Post Hoc test to assess statistical analysis for every individual pair in a group. P value less than or equal to 0.01 was considered statistically significant.

Result

Histological analyses

Microscopic examination of the rat's tongue of the saline/water and saline/honey groups revealed that the oral mucosa covering the underside of the rat's tongue being composed of keratinized stratified squamous epithelium. Besides, the lamina propria was generally thin, contained collagen fibers and small blood vessels. Groups of well formed striated muscles were also noticed beneath the lamina propria, and no inflammatory cells infiltration or congested blood vessels were seen (Figure 1, A1 and A2).

The MTX/water group showed that the ventral surface of the tongue revealed an obvious reduction in the thickness of the keratin and the epithelium, the supporting basement membrane was almost straight and congested blood vessels were detected in the underlying lamina propria. Vacuolar degeneration of some epithelial cells was also detected (Figure -1, B1 and B2).

The MTX/honey group showed increase in the thickness of the epithelium in comparison with the MTX/water group, the epithelium nearly restores its integrity and revealed marked improvement with the absence of congested blood vessels in the connective tissue (Figure -1, C1 and C2). The inflammatory infiltrates was not observed in all groups.

There was a statistically significant difference ($P < 0.01$) present between MTX / water group (19.22 ± 3.56) and the other three groups regarding the thickness of the epithelium, but a statistically no significant difference ($P > 0.01$) was observed between saline / water (43.8 ± 4.35) and saline/honey groups (44.2 ± 3.30), saline/water and MTX / honey groups (41.43 ± 2.99) or between saline / honey and MTX / honey groups.

There was a statistically significant difference ($P < 0.01$) present between MTX/water group (5.30 ± 0.22) and the other three groups regarding the number of congested blood vessels. A statistically no significant difference ($P < 0.01$) was observed between saline/water (1.10 ± 0.45) and saline/honey (0.8 ± 0.20), saline/water and MTX/honey groups (1.98 ± 0.80) or between saline/honey and MTX/honey groups (Table-1).

Immunohistochemical findings

Photomicrograph of the ventral tongue region mucosa of rats in the saline/water group and saline/honey group revealed moderate Ki-67 immuno reactivity in nuclei of cells of basal and supra basal cells layers, and mild positive immunohistochemical expression of Bcl-2 in the epithelial cells cytoplasm of basal and supra basal cells layers. The MTX / water group showing mild positive immune reaction of Ki-67 in some nuclei of basal epithelial cells, and negative immunohistochemical expression of Bcl-2 in the epithelial cells cytoplasm. The MTX / honey group showing mild positive immune reaction of Ki-67 in some nuclei of basal epithelial cells and mild immunohistochemical expression of Bcl-2 in the epithelial cells cytoplasm (Figure 2).

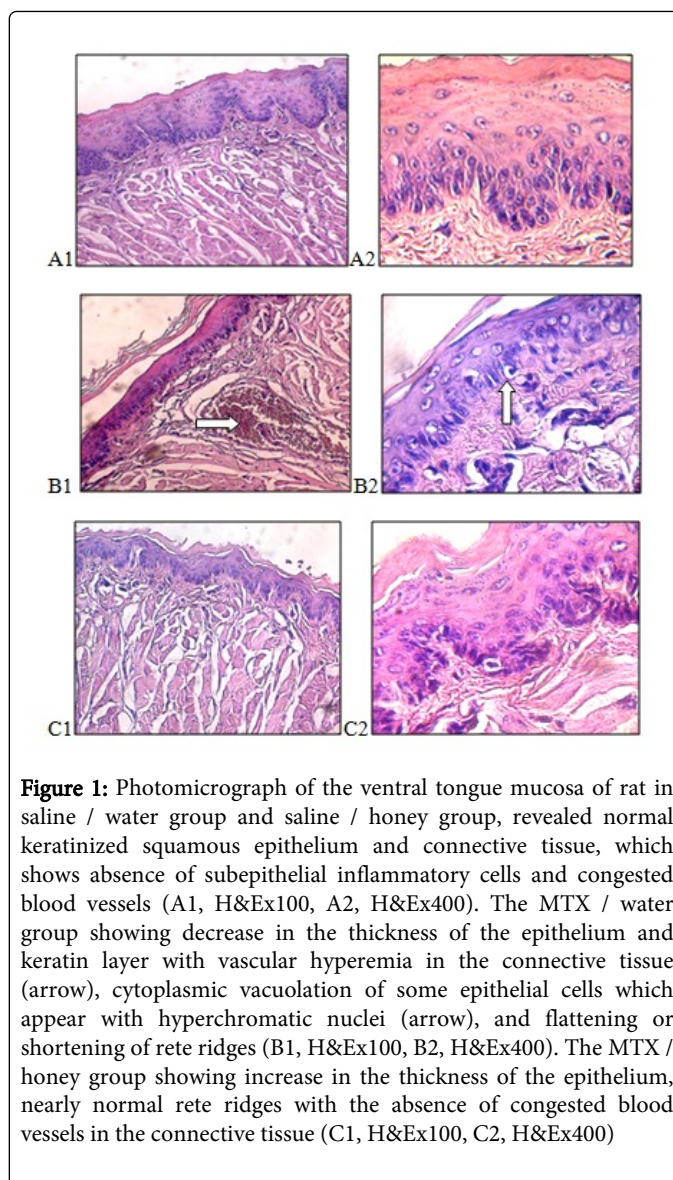


Figure 1: Photomicrograph of the ventral tongue mucosa of rat in saline / water group and saline / honey group, revealed normal keratinized squamous epithelium and connective tissue, which shows absence of subepithelial inflammatory cells and congested blood vessels (A1, H&Ex100, A2, H&Ex400). The MTX / water group showing decrease in the thickness of the epithelium and keratin layer with vascular hyperemia in the connective tissue (arrow), cytoplasmic vacuolation of some epithelial cells which appear with hyperchromatic nuclei (arrow), and flattening or shortening of rete ridges (B1, H&Ex100, B2, H&Ex400). The MTX / honey group showing increase in the thickness of the epithelium, nearly normal rete ridges with the absence of congested blood vessels in the connective tissue (C1, H&Ex100, C2, H&Ex400)

Statistical analysis showed that there was a statistically significant differences ($P < 0.01$) present between saline/water group (16.33 ± 0.24) or saline/honey group (17.14 ± 0.22) and the other two groups in terms of the rate of proliferation, but a statistically no significant differences ($P > 0.01$) was observed between MTX/water group (1.12 ± 0.10) and MTX / honey group (2.16 ± 0.35), and between saline/water group and saline/honey group.

The Bcl-2 immune staining results in the MTX/honey treated group showed mild positive Bcl-2 immunoreaction in the cytoplasm of some epithelial cells, but it was negative in the MTX/water treated group. There was a statistically significant difference ($P < 0.01$) present between MTX/water group (0.01 ± 0.02) and the other three groups in terms of the rate of anti-apoptosis. A statistically significant difference ($P < 0.01$) was also observed between saline/water (9.12 ± 0.50) and MTX/honey group (4.16 ± 0.22), saline/honey (9.98 ± 0.61) and MTX/honey group, but a statistically no significant difference ($P > 0.05$) was observed between saline/water and saline /honey group (Table-2).

Groups	Major epithelial thickness	P-value	Number of congested blood vessels	P-value
Saline/water	43.8 ± 4.35	0.565	1.10 ± 0.45	0.627
Saline/honey	44.2 ± 3.30		0.8 ± 0.20	
Saline/water	43.8 ± 4.35	0	1.10 ± 0.45	0
MTX/water	19.22 ± 3.56		5.30 ± 0.22	
Saline/water	43.8 ± 4.35	0.119	1.10 ± 0.45	0.327
MTX/honey	41.43 ± 2.99		1.98 ± 0.80	
Saline/honey	44.2 ± 3.30	0	0.8 ± 0.20	0
MTX/water	19.22 ± 3.56		5.30 ± 0.22	
Saline/honey	44.2 ± 3.30	0.129	0.8 ± 0.20	0.193
MTX/honey	41.43 ± 2.99		1.98 ± 0.80	
MTX/water	19.22 ± 3.56	0.009	5.30 ± 0.22	0
MTX/honey	41.43 ± 2.99		1.98 ± 0.80	

Table 1: Comparison between groups in the means and standard deviations of the histopathological parameters used in the study in rat's tongue mucosa following water or honey administration in female Albino rats post saline or methotrexate injection.

Discussion

Cancer patients receiving radiotherapy or chemotherapy for head and neck cancer usually suffers from oral mucositis which is considered as a severe complication, for which no effective intervention has been developed. Several studies were used topically applied honey for treatment of oral mucositis like Biswal et al. [15], Motallebnejad et al. [10], Rashad et al. [16], Khanal et al. [17], Abdulrhman et al. [18] and Sedighi et al. [19]. They showed that topically applied honey produced faster healing in patients with oral mucositis. However, despite using different types of honey, different scoring systems, and different inclusion criteria, all these trials showed about 50% decrease in the severity of oral mucositis in the honey arm. Parsons [20] found that there was no statistically significant difference in the severity of oral mucositis reported between those taking honey and those using standard forms of treatment.

All the patients reported that the honey was difficult to be tolerated once their mouth began to ulcerate, and patients found it difficult to manipulate the honey to coat all the oral mucosa. In addition to that, these studies did not address problems of tissue breakdown or impaired healing. For this reason, this study was conducted to found the systemic effect of honey on oral mucositis induced by MTX (60 mg/Kg) in rat model.

The present study showed that MTX cause a decrease in the thickness of the epithelium, vacuolar degeneration of epithelial cells, flattening or shortening of rete ridge, and vascular hyperemia.

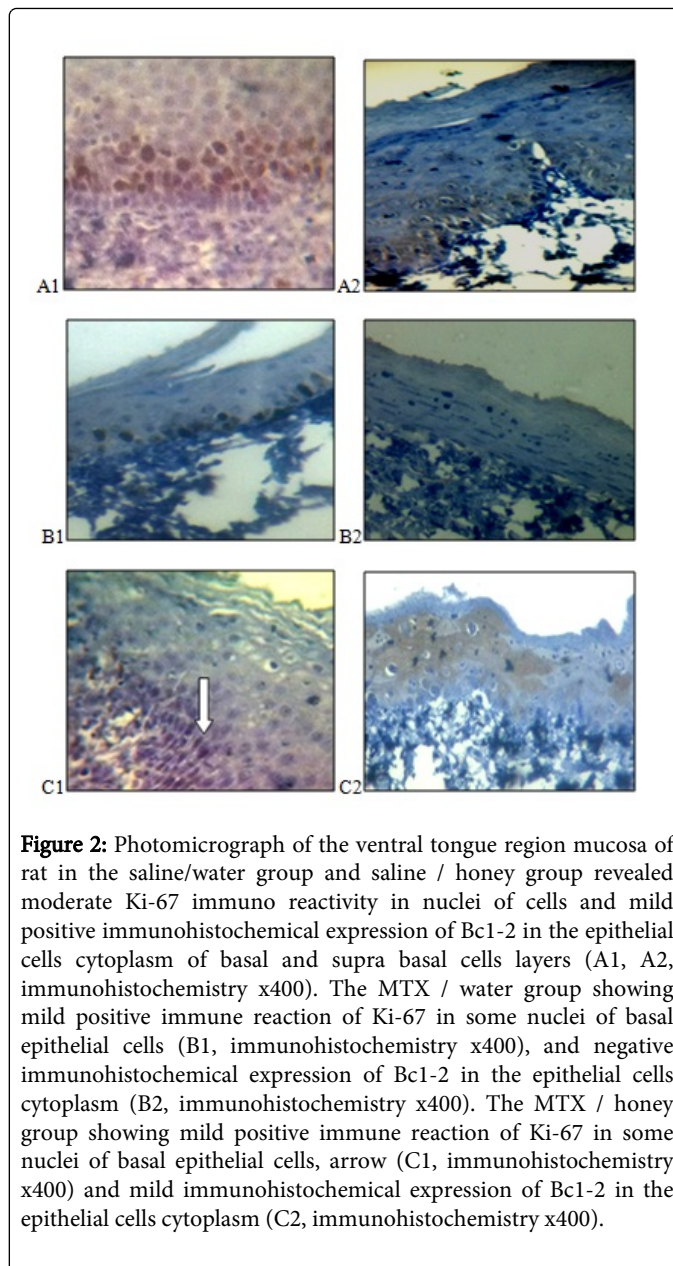


Figure 2: Photomicrograph of the ventral tongue region mucosa of rat in the saline/water group and saline / honey group revealed moderate Ki-67 immuno reactivity in nuclei of cells and mild positive immunohistochemical expression of Bc1-2 in the epithelial cells cytoplasm of basal and supra basal cells layers (A1, A2, immunohistochemistry x400). The MTX / water group showing mild positive immune reaction of Ki-67 in some nuclei of basal epithelial cells (B1, immunohistochemistry x400), and negative immunohistochemical expression of Bc1-2 in the epithelial cells cytoplasm (B2, immunohistochemistry x400). The MTX / honey group showing mild positive immune reaction of Ki-67 in some nuclei of basal epithelial cells, arrow (C1, immunohistochemistry x400) and mild immunohistochemical expression of Bc1-2 in the epithelial cells cytoplasm (C2, immunohistochemistry x400).

The stem cell population in the oral mucosa has a very high cell turnover rate. This rapid course of cell proliferation and constant epithelial replacement renders the mucosa susceptible to the effects of cytotoxic drugs that affect rapidly proliferating cells. A complex mechanism is involved in the pathophysiology of mucositis. Chemotherapy generates ROS which are deleterious to the DNA of epithelial cells. ROS may induce a cascade of biological events, which in turn result in the synthesis of various pro-inflammatory cytokines. These cytokines target epithelium, endothelium and connective tissue, thereby causing tissue injury [21]. These inflammatory mediators cause further damage either directly or indirectly by increasing vascular permeability, thereby enhancing cytotoxic drug uptake into the oral mucosa [22].

Munaretto et al. [23] immune suppressed the mice with the subcutaneous injections of 2.5 mg/kg of MTX for three consecutive

days, the epithelial thickness of the ventral surface of the tongue was increased significantly in the second day of the experiment, but it decrease gradually later on. The number of blood vessels and inflammatory cells per field in the connective tissue was similar in control and experimental samples. The differences between the present result and other results in the methodology used prevent a direct comparison between them.

The present study also showed that the use of honey was more effective in alleviating oral mucositis in comparison to placebo. It significantly increases the thickness of oral epithelium and decrease the number of congested blood vessels in comparison with the MTX/water treated group.

Groups	Ki-67	P-value	Bcl-2	P-value
Saline/water	16.33 ± 0.24	0.443	9.12 ± 0.50	0.195
Saline/honey	17.14 ± 0.22		9.98 ± 0.61	
Saline/water	16.33 ± 0.24	0	9.12 ± 0.50	0
MTX/water	1.12 ± 0.10		0.02	
Saline/water	16.33 ± 0.24	0	9.12 ± 0.50	0.007
MTX/honey	2.16 ± 0.35		4.16 ± 0.22	
Saline/honey	17.14 ± 0.22	0	9.98 ± 0.61	0
MTX/water	1.12 ± 0.10		0.01 ± 0.02	
Saline/honey	17.14 ± 0.22	0	9.98 ± 0.61	0.005
MTX/honey	2.16 ± 0.35		4.16 ± 0.22	
MTX/water	1.12 ± 0.10	0.022	0.01 ± 0.02	0.003
MTX/honey	2.16 ± 0.35		4.16 ± 0.22	

Table 2: Comparison between groups in the means and standard deviations of the immunohistochemical results in rat's tongue mucosa following water or honey administration in female Albino rats post saline or methotrexate injection.

Besides its sugar content, honey consists of several biologically active constituents such as vitamins, minerals, amino acids, proteins, as well as folic acids. This will help repair tissue directly [24]. Some of the flavonoids that have been identified in honey accelerate the healing process of oral mucositis by its anti-oxidant and free radical scavenging ability and it markedly suppressed the release of pro-inflammatory cytokines including TNF- α and IL-1 β [25,26]. In addition to honey beneficial role as anti-inflammatory, it also has been endorsed to its antimicrobial as well as boosting of the immune system [27]. The data indicate that honey can modulate the molecular processes of initiation, promotion, and progression stages [28].

Ki-67 is a nuclear proliferation-associated antigen expressed in the growth and synthesis phases of the cell cycle but not in the resting phase. This antigen provides information about the proportion of active cells in the cell cycle [29]. The bcl-2 gene encodes a protein located in the nuclear membrane, on the inner surface of mitochondria, and the endoplasmic reticulum. It is the most important gene of the bcl-2 family and has been reported to prolong the survival of cells by specifically inhibiting apoptosis. The balance between mitotic activity and apoptosis is thought to regulate normal development [30].

In the controlled groups, the Ki-67 expression was mostly seen in the basal and supra basal epithelial cells, this suggests that the epithelial basal and supra basal compartments have controlled proliferation rate but with a continuous proliferative capacity. A statistically no significant difference ($P > 0.01$) was present between saline/water and saline/honey group in terms of the rate of proliferation and antiapoptosis. This is because honey is non-cytotoxic to normal cells [31].

In the MTX/water group, the methotrexate causes significant reduction in the Ki-67 and Bcl-2 immune expression labeling indices in comparison with the control groups. MTX cause decreased DNA synthesis and cellular replication [32] and activates the apoptotic pathway [33].

In the MTX/honey group, the epithelium restores its integrity and revealed marked improvement, statistical analysis showed significant increase in Bcl-2 and no significant increase in Ki-67 immune expression labeling indices in comparison with the MTX/water group. The epithelial cells may respond more frequently with arrest or senescence than with apoptosis.

Conclusion

In the present study, the combined treatment of MTX and honey ameliorated the cytotoxic changes in the ventral tongue mucosa of rat induced by MTX alone. Honey cause significant increase in the epithelial thickness, significant decrease in the number of congested blood vessels, nonsignificant increase in the Ki-67 immune expression, and significant increase in Bcl-2 immuno expression in comparison with non-honey MTX treated group.

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