Original Research Article

Protective Role Of Honey on The Dorsal Surface of The Tongue of Methotrexate Treated Rats (Histological and Immunohistochemical Study)

Ali Sultan Al-Refai1*  Hassan Ali Al-Barazenchy1  Hanan Abdulla Abdulqader1
Ameera Kamal Khalili2

1College of Dentistry, Hawler Medical University, Erbil, IRAQ
2College of Dentistry, University of Duhok, Duhok, IRAQ

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Abstract
The most common side effect of chemotherapy is oral mucositis and the effective treatment is considered important in cancer patient. This study was aimed to evaluate the effect of honey as a treatment for dorsaltongue mucositis caused by the methotrexate.
Forty Albino rats were used in the present study and grouped randomly into control and study groups(20 animals each). The rats in the control group were divided randomly into two groups: 10 animals were treated by distilled water and 10 animals were treated by (2.5gm/kg) honey two times daily for eight days using gavage needle, and a physiological saline was intraperitoneally injected at day four for all the animals. In the study group, they were intraperitoneally injected by 60 mg/kg of methotrexate at day four and the animals were treated in the same way like control group. At day eight, all the animals were sacrificed;and a cross section from the tongue was removed for histopathological and immunohistochemical (Ki-67 and Bcl-2 immunolabeling) analysis.

Honey can partially protect the tongue from methotrexate induced cytotoxicity, and attenuate the associated injury. Ki-67 immune expression was non significantly increased (p>0.05), but Bcl-2 immune expression was significantly increased in (p<0.05) in comparison with methotrexate treated group.

Honey can produce protection against methotrexate induced dorsal tongue mucositis.Flavonoids and phenolic compounds in honey can accelerate the healing process by its anti-oxidant and free radical scavenging properties.

Key Words: Honey, Mucositis, Chemotherapy, ki-67, Bcl-2.

الخلاصة

الأثار الجانبية الأكثر شيوعا للعلاج الكيميائي هي التهاب الغشاء المخاطي بالفم والعلاج الفعال له يعتبر مهمة لمرضى السرطان. تهدف هذه الدراسة إلى تقييم تأثير العسل كعلاج لالتهاب الغشاء المخاطي لللسان الناجم عن الميثوتريكتس.

استخدم أربعين جرذ في الدراسة الحالية، وتم تقسيم أربعين حيوانا إلى مجموعتين: دراسة ودراسة. أجريت دراسة (20 حيوانا لكل منها). تم تقسيم الفئران في المجموعة الضابطة إلى مجموعتين وعينت 10 حيوانا على خلفية الماء الفضي. والживانات عولجت ب (2.5 غم/كغ) من العسل مرتين يوميا لمدة ثمانية أيام باستخدام إبرة أنبوب تغذية، أما المجموعة الضابطة، اما مجموعة الدراسة، فقد تم حفظها بنسبة 60 ملغ/كم من الميثوتريكتس في اليوم الرابع والحيوانات عولجت بنفس الطريقة مثل مجموعة الضابطة. في اليوم الثامن، تم التصوير بجميع الحيوانات. وتمت إزالة شريحة عرضية من الفم لأغراض الدراسة التحليلية المرضية والمناعية (Ki-67 and Bcl-2 immunolabeling).

العمل يمكن أن يحمي جزئيا اللسان من السمى الخلوية الناجمة من الميثوتريكتس ويعضف التأثير المزمن به التعبير المناعي إلى 67-Ki، ويظهر زيادة غير مميزة ملحوظة (p<0.05)، ولكن التعبير المناعي إلى 2-Bcl معنوية (0.05) مع التغيير المزمن (Ki-67) مع ارتفاع مستوى اللدون معدل مع الفئران من العسل يمكن أن ينتج جملة ضد التهاب الغشاء المخاطي الظهري اللسانى الناجم عن الميثوتريكتس. مركبات الفلافونويد ومركبات الفينول في العمل يمكن أن ينتج شفاء بواسطة مضادات الأكسدة وخصائص كسم الجذور الحرة.
Mucositis is a form of mucosal injury and considered as one of the most common complication for cancer treatment [1]. Methotrexate is an antimetabolite and one of the folic acid antagonists; it represents one of the most potent classical anti-tumor drugs, that causing reduction in DNA formation and cell proliferation[2]. Oral mucositis usually affecting most of the patients who taken chemotherapy and almost all those undergoing radiotherapy[3].The complications sometimes result in discontinuation orchemotherapy treatment interruption [4].

Histopathologically, oral mucositis lesions involve the epithelium causing decrease in epithelial thickness by apoptosis and then affect the connective tissue [5]. The oral cavity has a variety of microorganisms; this may leads to a marked increase in the risk of bacteremia and sepsis [6], and serious complications such as difficulty in eating and swallowing, mouth pain, infection, malnutrition, weight loss and thus patient survival [7,8].

Honey is one of the nature’s wonders. It is a saturated solution of sugar that is made from nectar and sweet deposit collected from flowers by bees and stored in honeycombs. A ripening process follows where the invertase enzyme convert the sucrose into glucose and fructose and then the overall water content is reduced [9]. The composition of honey is depends on the geographical and floral source of the nectar. Honey contains organic acids, enzymes, amino acids, vitamins, calcium,zinc, iron, chromium, manganese and different inorganic substances. It also contains a blend of flavonoids and phenolic acid, which are antioxidants that eliminate the free radicals in the human body [10]. In recent times honey has been used to treat different types of surgical wounds, burns, gastric and diabetic ulcers [11], and radiation-induced oral mucositis in cancer patients [12].

The dorsal tongue mucosa is a keratinized stratified squamous epithelium which contains different types of papillae [13]. The aim of the present study was to investigate the effects of intraperitoneal injection of methotrexate (60) mg/kg on rat’s tongue mucosa, and to evaluate the preventive role of honey for the methotrexate induced dorsal tongue mucositis. Histological and immunohistochemical investigations were used to clarify its effect on cell proliferation and cell apoptosis.

### Materials and Methods

Forty females Albino rats, weighing (260-290) g were cared in the animal house of College of Medicine/Hawler Medical University, Erbil/Kurdistan Region of Iraq, and maintained on a 12 hour light and dark cycle at 24± 6°C. They were allowed to drink water and fed with a standard rat chowad libitum. Under protocol, the research project was approved by the Research Ethics Committee/College of Dentistry/Hawler Medical University.

Fifty grams of natural unprocessed honey was obtained from Mergasour / Iraq and dissolved in (40) ml of distilled water (every one ml contain 1.25g honey) and the mixture was filtered with a fine muslin cloth and then with What man filter paper (no.1).

Some authors[14,15] found that the clinical signs of mucositis were present in rats treated by the(60) mg/kg of methotrexate, and started from the second day until the fifth day of the experiment, and the symptoms of the induced mucositis were most severe in the fourth day of the experiment, and after that the rats started gradually to recover. For this reason the animals were sacrificed four days after intraperitoneal injection of methotrexate.

Forty Albino rats were used in the present study and grouped randomly into control and study groups (20 animals each). The rats in the control group were divided into two groups: ten animals were treated bya volume of distilled water equal to honey, and ten animals were treated by (2.5gm/kg) of honey [16] two times daily, the treatment continue for eight
days using gavage needle and a physiological saline in a similar dose of methotrexate was intraperitoneally injected at day four. In the study group, they were intraperitoneally injection by (60) mg/kg of methotrexate at day four and the animals were treated in the same way like control group.

All the animals were sacrificed at day eight, a cross section of the middle third of the tongue was removed, processed for routine hematoxylin and eosin and for immunohistochemical analysis using Ki-67 immunohistochemistry to assess the cell proliferation, but the expression of anti-apoptotic protein was assessed by Bcl-2 immunostaining.

Monoclonal mouse anti-human Ki-67 antigen, code No. M 7240, clone MIB-1 staining system, and Bcl-2 oncoprotein, code number 1587, clone 124 ready to use N-series primary antibody, use with Dako EnVision™, EnVision™ double staining and LASAB™ 2 systems. Positive and negative controls were used in the study and run simultaneously with all biopsy specimen. Ki-67 positive cells were identified by brown nuclei, while Bcl-2 positive cells was demonstrated brown cytoplasmic staining. The evaluation was carried out by two observers. Five sections were randomly chosen for each specimen using a light Olympus microscope, and approximately 1000 cells at high power field (x400) were counted and the percentages of Ki-67 and Bcl-2 positive cells were calculated.

Morphometric analysis used to measure the thicknesses at high power magnification (x400) of all layers of the epithelium which include stratum basale, stratum spinosum, and stratum granulosum in five photograph fields from each tongue mucosal epithelium section using objective micrometer. The levels of Ki-67 and Bcl-2 expression were scored as absent: < 1%, mild: 1 - 10%, moderate: >10 - 50%, and strong: > 50% [17].

**Statistical Analysis**

Statistically, ANOVA test was used for comparison between means among groups for quantitative observations, and when it revealed that there was a statistically significant difference; Post-hoc Bonferroni test was performed to assess the individual pair of groups for statistically significant finding. P value less than or equal to 0.05 was considered statistically significant.

**Result**

The microscopical picture of dorsal tongue mucosain animals which were intraperitoneally injected by saline and treated by water (S/W group) showed normal appearance and structure. The dorsal surface of tongue exhibited sharp conical projections of filiform papillae with pointed tips (Figure -1, A). All types of papillae are regular in height, shape, distribution, and orientation with smooth regular keratinized epithelial covering. The connective tissue papillae appear normal. The skeletal muscle fibers are seen underneath the papillae and running in different directions. The dorsal surface of tongue in animals which were intraperitoneally injected by saline and treated by honey (S/H group) showed the same microscopic features.

The dorsal surface of tongue in animals which were intraperitoneally injected by methotrexate and treated by water (M/W group) reveled loss of the normal appearance and shape of the filiform and fungiform papillae (Figure -1, B1, B2). Most of the filiform papillae showed loss of their characteristic conical pointed shape and were missing in some areas. The keratin layer showed separation and vacuolation of the basal and supra basal epithelial cells layers were also seen.

The dorsal surface of tongue in animals which were intraperitoneally injected by methotrexate and treated by honey (M/H group) showed nearly restoration of normal shape of papillae, and the filiform papillae appear conical with pointed tips. The keratin layer showed also separation from underlying epithelial cells and some vacuolated epithelial cells were also still present (Figure-1, C1, C2).
Connective tissue inflammatory cell infiltration was not observed in all groups. Regarding the epithelial thickness, ANOVA test showed a significant differences (p=0.000) present between the four studied groups. In the control groups, the mean epithelial thicknesses in the S/W and S/H treated groups were 49.28±1.53 and 51.66±1.98 respectively. Statistical analysis showed that there were no significant differences present between these two groups regarding the epithelial thickness (p=0.288). So the S/W group was considered the reference one for the control groups. In the study groups, the mean epithelial thicknesses in the M/W and M/H treated groups were 25.87 ± 2.39 and 36.35 ± 2.32 respectively. Statistical analysis showed that there were significant differences present between M/W and the S/W (p=0.000), M/W and M/H treated groups(p=0.000) regarding the epithelial thickness.

Regarding Ki-67 and Bcl-2 immune expressions, ANOVA test showed a significant differences (p=0.000) present between the four studied groups. Immunohistochemistry results of the dorsal tongue epithelium of the control and experimental groups are expressed in Table -1. Photomicrograph of the dorsal tongue mucosa of rats in the S/W and S/H groups revealed moderate Ki-67 immuno-reactivity in the nuclei of the basal cells layer and some supra basal layers. Mild positive immunohistochemical expressions of Bcl-2 in the cytoplasm of some epithelial cells were also seen. Statistical analysis showed no significant differences present in the number of Ki-67 and Bcl-2 positive cells between S/W and S/H groups (p>0.05).

The number of cells staining positive for Ki-67 andBcl-2 in the M/W and M/H groups showed a significant decrease (p<0.05) in comparison with the control groups. The M/W and M/H groups showed a negative immune expression of Ki-67 with a non-significant increase in the M/H group (p>0.05). The Bcl-2 immune staining expression results in the M/H treated group showed mild positive Bcl-2 immune reaction in the cytoplasm of some epithelial cells, but it was negative in the M/W treated group. The treatment with honey cause significant increase in the number of Bcl-2 positive cells in the M/H group in comparison with the M/W group (p<0.05) as seen in Table-1.

Figure-2 shows negative immune reactivity to Ki-67 in the nuclei of epithelial cells of the dorsal tongue epithelium in the M/W and M/H treated groups. While Figure-3 shows negative immune reactivity to Bcl-2 in the dorsal tongue epithelium of M/W treated group and mild Bcl-2 immune reactivity in the dorsal tongue epithelium in the M/H treated group.
Table 1: Immunohistochemical results of the tongue following water or honey treatment in female Albino rats post saline or methotrexate injection of all groups in the study.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ki-67</th>
<th>P-value</th>
<th>Bcl-2</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>S/W</td>
<td>11.53± 0.33</td>
<td>0.495</td>
<td>5.001 ± 0.96</td>
<td>0.069</td>
</tr>
<tr>
<td>S/H</td>
<td>11.76±0.48</td>
<td></td>
<td>6.01 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>S/W</td>
<td>11.53± 0.33</td>
<td>0.000</td>
<td>5.001 ± 0.96</td>
<td>0.000</td>
</tr>
<tr>
<td>M/W</td>
<td>0.032 ± 0.01</td>
<td>0.02±0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/W</td>
<td>11.53± 0.33</td>
<td>0.000</td>
<td>5.001 ± 0.96</td>
<td>0.000</td>
</tr>
<tr>
<td>M/H</td>
<td>0.032 ± 0.01</td>
<td>0.02±0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/W</td>
<td>11.76±0.48</td>
<td>0.000</td>
<td>6.01 ± 0.04</td>
<td>0.000</td>
</tr>
<tr>
<td>M/W</td>
<td>0.000</td>
<td>0.768</td>
<td>0.02±0.01</td>
<td>0.000</td>
</tr>
<tr>
<td>S/H</td>
<td>11.67±0.48</td>
<td>0.000</td>
<td>6.01 ± 0.04</td>
<td>0.000</td>
</tr>
<tr>
<td>M/H</td>
<td>0.032 ± 0.01</td>
<td>0.02±0.01</td>
<td>2.05 ± 0.21</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Figure 1: The dorsal surface of the tongue in the S/W treated groups exhibited sharp regular filiform papillae with pointed tips (A, H&E x100). But the M/W treated group revealed loss of the conical shape of filiform papillae with separation of keratin layer, and the fungiform papillae showed atrophic changes. The epithelium contains shallow epithelial ridges and showed vacuolar degeneration, arrow (B1, H&E x100; B2, H&E x400). The dorsal surface of the tongue in the M/H treated group showed that some of the filiform papillae restore its conical shape, the shape of the filiform and the fungiform papillae with its taste bud appear nearly similar to normal but with some cellular vacuolation and separation of keratin layer (C1, H&Ex100, C2, H&Ex400).
Figure 2: Moderate Ki-67 immuno-reactivity in nuclei of basal and supra basal cells layer seen in the S/W treated group (A, immunohistochemistry x400). Negative immune reactivity to Ki-67 in the tongue epithelium of M/W treated group (B, immunohistochemistry x400) and the M/H treated group (C, immunohistochemistry x400).

Figure 3: Mild cytoplasmic reaction to Bcl-2 in tongue epithelium of the S/W treated group (A, immunohistochemistry x400). Negative immune reactivity to Bcl-2 in the tongue epithelium of M/W treated group (B, immunohistochemistry x400). Mild Bcl-2 immuno-reactivity in the tongue epithelium in the M/H treated group, (C, immunohistochemistry x100).

Discussion

Oral mucositis is considered a debilitating complication in cancer patients receiving radiotherapy or chemotherapy [18]. Nowadays, the research activity are focusing on finding anatural pharmacological compound which can be used for treatment of oral mucositis, but no effective intervention has been developed[19], due to the complex biological cause of oral mucositis [20]. The treatments used in previous studies can improve patient comfort, but they do not address the problems of tissue breakdown or impaired healing [21].

One of the interventions for the management of radiation-induced oral mucositis is topically applied natural honey. Some authors [12, 22, 23] conducted a clinical trial investigating the effect of honey on oral mucositis in patients receiving radiation therapy, and reported a significant reduction in the severity of oral mucositis. Some studies [24, 25] found that topically applied honey produced faster healing in patients with chemotherapy-induced oral mucositis. Parsons [26] found that honey showed no statistically significant effect in treatment of radiotherapy induced oral mucositis. All patients reported a severe decrease in quality of life throughout the course of their radiation therapy, irrespective of whether they were taking honey or not. The patients found it is very difficult to manipulate the honey and coating the oral mucosa; in addition, these studies did not address
histological changes like tissue breakdown and impaired healing. For this reason, we analyzed the effects of honey on methotrexate induced oral mucositis in Albino rat tongue papillae.

Decrease in the thickness of the epithelium, epithelial cells vacuolar degeneration, and shortening or flattening of rete ridges were seen associated with the use of methotrexate. Reactive oxygen species is generated by methotrexate which is deleterious to the DNA of epithelial cells and induce a cascade of biological events such as activation of nuclear factor-kappa B, and in turn result in the synthesis of cytokines which is responsible for tissue injury [27].

The current study also showed that honey can decrease the severity of oral mucositis in comparison to placebo treatment. Its vitamins, enzymes, and minerals contains, in addition to its antimicrobial property can accelerate the tissue repair directly [28]. Flavonoids and phenolic compounds in honey can accelerate healing by its antioxidant activity and free radical scavenging properties [29]. Honey can markedly suppress the release of pro-inflammatory cytokines like the TNF-α and IL-1β [30] and can modulate all the molecular processes of oral mucositis like initiation, promotion, and progression stages [31].

Ki-67 is a nuclear proliferation-associated antigen which provides the information about the proportion of active proliferating cells [32], but the bcl-2 gene has specifically inhibiting the apoptosis and prolongs the survival of cells. The balance between both can regulate the normal cells development [33]. The present results showed that the Ki-67 expression in the controlled groups was mostly seen in the basal and supra basal epithelial cells layer, this is due to a continuous controlled proliferative capacity rate. A statistically no significant difference (P>0.05) was present between S/W and S/H group in terms of the rate of proliferation and anti-apoptosis. Hussein et al found that honey is non-cytotoxic to normal cells [34].

In the M/W group a reduction in the Ki-67 and Bcl-2 immune expression labeling index was noticed significantly. Methotrexate causes decreased in DNA synthesis and cellular proliferation [35] and activates the apoptotic pathway [36]. In the M/H group, the tongue dorsal epithelium showed marked improvement. Statistical analysis showed a non-significant increase in Ki-67 and a significant increase in Bcl-2 immune expression labeling indexes in comparison with the M/W treated group. The epithelial cells maybe responding more frequently with cell arrest or senescence than with cell apoptosis.

Conclusion
The systemic use of natural honey produced protection against the methotrexate induced tongue mucositis and can accelerate the healing process.

References
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