Evaluation of the Possible Ameliorative Effect of camel milk against Toxicity Induced by Mono Sodium Glutamate on the dorsal surface of the tongue in Albino Rats

Abstract

Introduction: Monosodium glutamate (MSG) is a very well-known and commonly used food additive. Despite widespread use, MSG has raised safety concerns. It has countless harmful effects on different body organs. Camel milk (CM) has several health benefits, including antiviral, antibacterial, anticancer, and antioxidant properties, making it a potential mitigator of MSG toxicity. The aim of the study was to detect the possible protective effect of (CM) against MSG-induced structural alterations on the tongue's dorsal surface in albino rats. **Materials and Methods:** Thirty rats were randomly and equally divided into three groups (n=10). Group 1 (Control): Rats received saline by oral gavage (OG) for 40 days. Group 2 (MSG): Rats received 60mg/kg MSG via OG daily for 30 days. Group 3 (CM and MSG): Rats were administered CM via OG at a dose of 16.6 ml/kg/day for 10 days as a prophylactic dose prior to and then during MSG administration. Tongue specimens were examined histologically, immunohistochemically for inducible nitric oxide synthase (iNOS), and by qRT-PCR for heme oxygenase-1 (HO-1) gene expression.

Results: This study revealed that the MSG treated group showed marked signs of degeneration of the filiform, fungiform and circumvallate papillae of the dorsal surface of the tongue. It significantly increased iNOS immunoexpression and significantly downregulated (HO-1) gene expression level. The group co administered MSG with CM showed improvement of the histological picture, reduced the immunoexpression of iNOS and upregulated HO-1 gene expression.

Conclusion: CM has a potential protective antioxidant effect against the deleterious alterations caused by MSG induced toxicity.

Keywords: Camel milk, HO-1 gene, iNOS, Monosodium glutamate, Tongue.

1. Introduction

Mono sodium glutamate (MSG) is a very distinguished widely used food additive worldwide as a flavor enhancer. It is found in various food products, including processed meat, crackers, salad dressings, dietary supplements, even infant formula and vaccines. The global demand for MSG is huge, especially in Asian countries, with China having the highest consumption rate.[1]

Questions were raised about the safety of MSG, despite its widespread use even at low doses. Fast food containing MSG can cause symptoms such as weakness, flushing, dizziness, numbness, sweating, and headache. Moreover, further complications include asthma, urticaria, atopic dermatitis, abdominal discomfort, and neuropathy.[2] The deleterious effects of MSG on various tissues include the male reproductive system, placenta, kidneys, and liver. [3] Moreover, the damaging impact of MSG was also investigated in oral tissues, including the gingiva, buccal mucosa[4], and submandibular salivary glands.[5]

Oxidative stress can result from an inequity between the production of reactive oxygen species (ROS) and the body's antioxidant defenses. MSG-induced oxidative stress disrupts critical antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), thereby impairing the antioxidant shield system and exacerbating cellular damage. [6]

Multiple approaches were used to counteract the effects of MSG using natural and chemical antioxidant agents. Recently, there has been significant interest in identifying antioxidants from natural sources to inhibit the chain reaction of oxidation and retard the progression of many chronic diseases. [7]

Camel milk (CM) has multiple health benefits, including antiviral, antibacterial, antifungal, anticancer, and antioxidant effects. Moreover, it can regulate blood sugar levels and reverse signs of aging. It is used to treat high blood pressure, jaundice and asthma. [8] People in deserts and extremely dry regions refer to CM as 'white desert gold' because of its health benefits, given its high consumption rate. The composition of CM may vary based on many factors, including feeding conditions, geographic location, genetics, and health status. Its beneficial antioxidant effect might be due to 6.7 times more vitamin C in fresh CM than in cow milk, along with other antioxidant components such as caseins and lactoferrin that help in reducing oxidative stress.[9]

According to previous studies, this study aimed to investigate the possible mitigating effect of CM, a potent antioxidant, against toxic effects of MSG on the dorsal surface of the tongue in albino rats.

2. Materials and methods

2.1 Materials

Mono Sodium Glutamate (MSG) cat. No. ($C_5H_9NO_4$) in the form of crystal powder, purchased from the local market and licensed by the manufacturer Ajinomoto Co., Inc., Tokyo, Japan. The solution was prepared by adding 240g to 1 L. of distilled water. Then, each rat received a weight-correlated dose (60 mg/kg/day). Fresh CM was purchased raw in bottles. It is composed of 83-90% water and the rest dry matter including protein, fat, lactose and ash. It was purchased from a local farm (Tayyiba farm) weekly throughout the experiment duration and stored in the refrigerator. The recommended dose was 16.6 ml per day.

2.2 Methods:

2.2.1 Sample size calculation

The sample size for this study was calculated to be 30, according to Charan and Biswas (2013).[10]The following equation was used to detect the sample size: $N = (\boldsymbol{Z\alpha})\mathbf{2} * (\boldsymbol{S})\mathbf{2} \ (\boldsymbol{d})\mathbf{2}$ N = Total sample size, Zα= Standard normal variation, and it is equal to 1.96 at $P < 0.05$, SD = Standard deviation of the variable, d = Absolute error or precision

2.2.2 Study design:

After the approval of Research Ethics Committee (REC) of the Faculty of Dentistry, Suez Canal University (number: 472/2022), thirty adult male albino rats with an average of 150-200 grams body weight were housed in rat cages, labeled with numerical numbers and kept in well-ventilated animal house of the Faculty of Dentistry, Suez Canal University. The rats were kept at 27-30°C, with 12 hours of natural light, and fed dry pellets and water *ad libitum*.

Thirty rats were randomly divided into three groups (n=10) as follows: Group 1 received distilled water daily via oral gavage (OG) for 40 days till the end of the experiment. [11] Group 2 received 60 mg/kg MSG daily for 30 days via OG.[12] Group 3 received 16.6 ml/kg CM for 10 days as a prophylactic measure followed by a combination of MSG and CM for 30 days via

OG.[13]After euthanasia, the tongue specimens were dissected. The dead rats were disposed of by burning in the Animal Aching Unit of Medicine, Suez Canal University.

2.2.3 Histopathological Analysis

After separating the specimens, half were fixed in 10% buffered formalin and embedded in paraffin. Examination of 5 μm thickness of hematoxylin and eosin-stained sections were mounted for examination by ZEISS Primo Star light microscope and photographed using a Tucsen IS 1000 10.0MP camera in the Oral Biology lab, Faculty of Dentistry, British University in Egypt.

2.2.4 Immunohistochemical Analysis

Another 5 μ sections were cut and mounted on positively charged glass slides. Inducible nitric oxide synthase (iNOS) 'rabbit polyclonal antibody' was used according to the instructions of the manufacturer for ROS identification. It was purchased from Thermo Fisher Scientific, Anatomical Pathology, Tudor Road, Manor Park, Runcorn, Cheshire WA7 ITA, UK (Catalog No. RB-9242-R7), with brown cytoplasmic and nuclear expression. The screen area was measured from digital images acquired at 400× magnification. The area percentage was quantitatively assessed using ImageJ analysis (Fiji distribution) in the Pathology Laboratory, Faculty of Medicine, Suez Canal University.

2.2.5 Molecular Analysis

The remaining half of the tongue specimens were kept at -80°C for real-time quantitative polymerase chain reaction (qRT-PCR). For the detection of Heme Oxygenase-1 (HO-1) gene, tissue homogenization was performed, followed by RNA extraction and purification using the RNeasy Mini kit (cat no: 74104). The reverse transcription step for cDNA synthesis by the reverse transcription kit, cat. No: 205310 was performed, then the HO1 gene amplification using primer assay cat no: 249900, PCR kit cat no: 204141, and β-actin cat no: 249900. Consequently, the real-time cycler was programmed for denaturation at 94ºC for 15 seconds, annealing at 55ºC for 30 seconds, and extension at 70ºC for 30 seconds, for 40 cycles. This investigation was conducted at Global Med Lab in Cairo, Egypt.

2.2.6 Statistical analysis

The numerical data were tabulated and analyzed for normality by calculating the mean ± standard deviation and evaluating histograms and normality curves using the Social Science Statistics software program version 23 (SPSS, Inc., Chicago, IL, USA). One-way ANOVA and Tukey tests were used to compare data on the percentage of positive reaction areas for iNOS and HO expression. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Histopathological Results:

Group 1 revealed normal mucous membrane of the tongue's dorsal surface. The filiform papillae appeared well-organized, conical, and coated with keratinized stratified squamous epithelium. The fungiform papillae had rounded, mushroom-shaped bulges with flattened tops with a single taste bud centrally located on top. The trough of the circumvallate papillae (CVP) was surrounded by taste buds and covered by stratified squamous epithelium. The lingual salivary glands consisted of serous and mucous acini with typical architecture. There was normal underlying connective tissue with no signs of inflammatory infiltrate **(Figure 1 A:D).**

Group 2 showed severe atrophic and degenerative changes within the mucous membrane of the dorsum of the tongue. The filiform papillae showed lost their characteristic conical shape with marked nonuniform orientation. Some areas showed keratin detachment from the underlying epithelium, as well as areas of hyper-keratinization with cytoplasmic vacuolations within the basal and supra-basal epithelial cells. The fungiform papillae were disfigured. The taste buds were deformed with an ill-defined taste pore. The CVP troughs and their taste buds showed deformities in their general outlines, with an undetected taste pore. The epithelium at the base of the trough lost its normal orientation. The lingual salivary glands showed marked atrophic changes in their acinar cells with loss of their typical architecture. There was marked cystic degeneration, acinar contraction, and widening of CT septa. The collagen fibers of the underlying lamina propria revealed signs of inflammation with marked dilatation of the blood vessels (**Figure 1 E:H).**

Group 3 showed marked improvement within the dorsal surface of the tongue that was coated by a normal thickness of keratinized stratified squamous epithelium. The filiform papillae nearly restored their pattern with tapering ends. The covering keratin layer appeared normal with

minor areas of separation. The fungiform papillae presented as almost normal, like those of the control group. Their taste buds revealed minimal signs of deformation or degeneration. The trough of the CVP retained its normal outline. The number and arrangement of taste buds on the sides of the trough appeared normal. The lingual salivary glands showed marked improvement of their acinar architecture with almost no cytoplasmic vacuolations and minimal cystic degeneration. The underlying connective tissue revealed mild inflammation with small blood vessels **(Figure 1 I: L).**

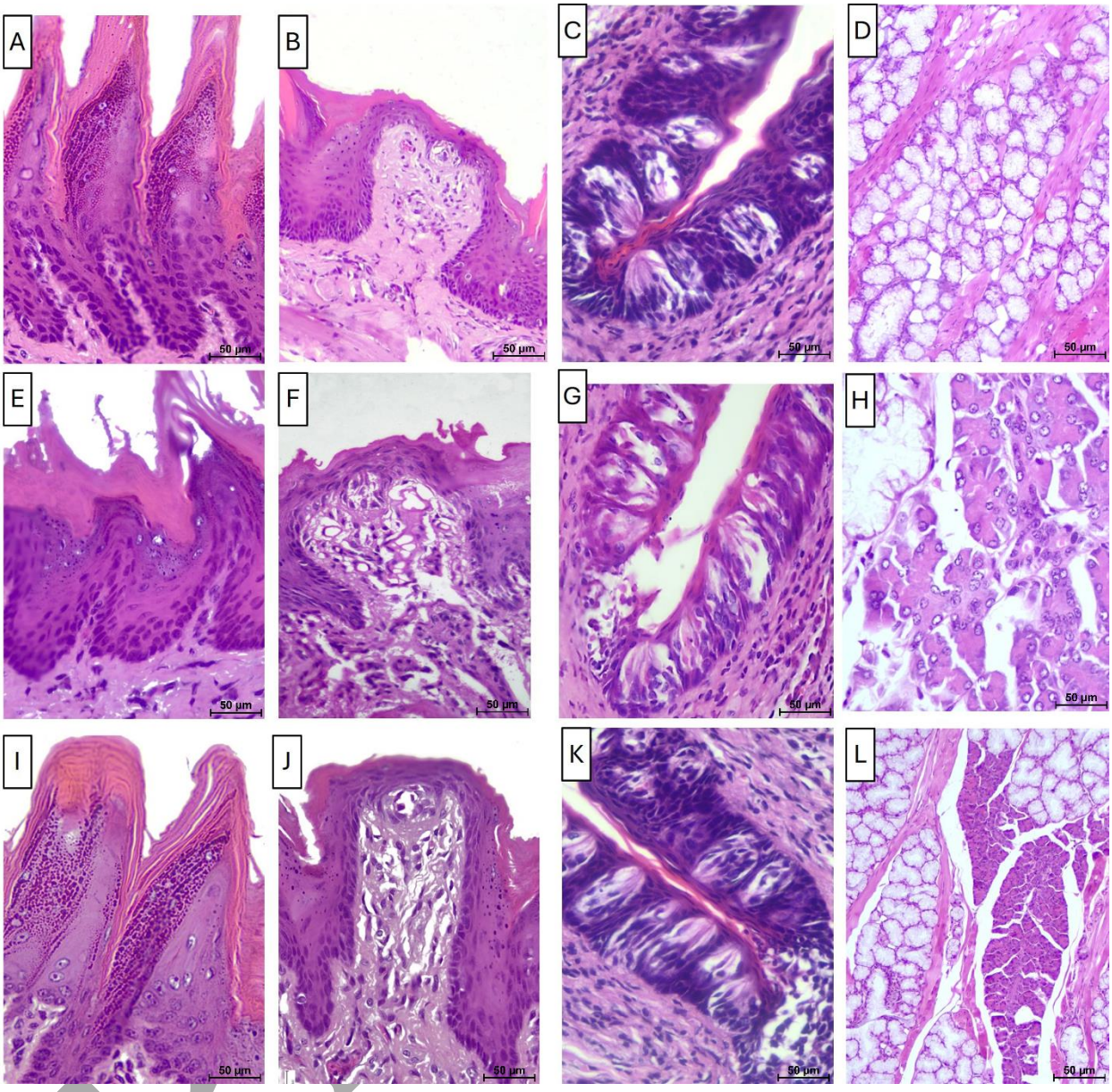

Figure 1: Photomicrographs of H & E-stained sections of the tongue's dorsal surface in different groups. Group 1 showing normal appearances of (A) filiform, (B) fungiform, (C) trough of circumvallate papilla, and (D) salivary gland architecture. Group 2 showing severe degenerative changes in (E) filiform, (F) fungiform, (G) trough of circumvallate papilla, and (H) salivary gland architecture. Group 3 showing marked improvement within (I) filiform, (J) fungiform, (K) trough of circumvallate papilla, and (L) salivary gland architecture. (H&E. orig. Mag. D, H, L 200X –A, B, C, E, F, G, I, J, K 400X)

3.2 Immunohistochemical Results

Examination of iNOS immunohistochemical expression in stained sections of the dorsal surface of the tongue in different groups showed variable results. Group 1 showed mild cytoplasmic immunoreactivity to iNOS (7.893 ± 0.4) allover epithelial thickness, represented by filiform papillae (Figure 2A). Group 2 showed strong cytoplasmic immunoreactivity for iNOS (68.06 ± 4.1) throughout the epithelial thickness, compared with the control group (Figure 2B). Group 3 revealed a marked decrease in the iNOS expression (17.34 ± 2) along the epithelial layers with moderate expression in the prickle cell layer (Figure 2C). Tukey's post hoc pairwise comparisons confirmed the ANOVA results, as shown in tables 1 & 2. Group 1 exhibited the lowest mean area percentage ($p < 0.05$). Group 2 showed a significantly higher mean area percentage than the other groups ($p < 0.05$). Group 3 showed moderate mean area percentages, which were higher than those of group 1 significantly ($p < 0.05$) but lower than those of group 2 **(Figure 2D)**.

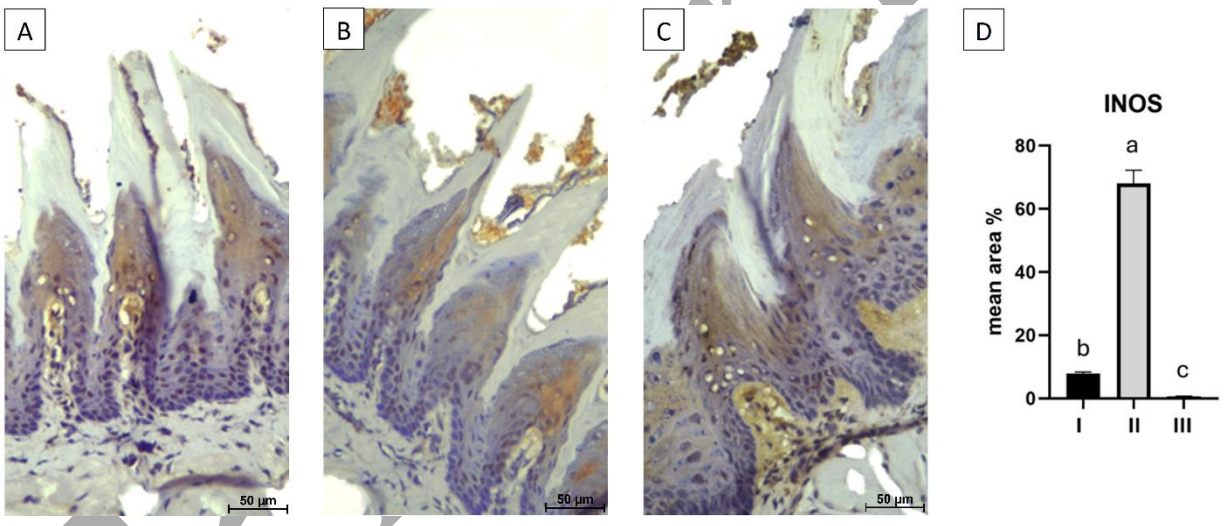

Figure 2: Photomicrographs showing iNOS immunohistochemical expression of the tongue's dorsal surface in different groups. (A) Group 1 showing mild cytoplasmic immunoreactivity. (B) Group 2 showing strong cytoplasmic immunoreactivity all over the epithelial thickness. (C) Group 3 showing moderate expression within the prickle cell layer. (orig. mag. 400X) (D) Histogram showing the mean area % of iNOS immunohistochemical expression.

3.3 Statistical results:

3.3.1 Mean area percentage of iNOS immunohistochemical expression:

One-way ANOVA showed a statistically significant difference in iNOS mean area percentage among the groups ($p < 0.0001$). Tukey's post-hoc pairwise comparisons demonstrated significant differences among all pairwise comparisons. Relative to Group 1, Group 2 showed a markedly higher iNOS mean area percentage (mean difference [Group I - Group II] = -60.16; 95% CI: -65.83 to -54.49; adjusted $p < 0.0001$). Group 3 was also significantly higher than Group 1 (mean difference [Group I - Group III] = -9.443; 95% CI: -15.11 to -3.772; adjusted $p = 0.0013$), but significantly lower than Group 2 (mean difference [Group II - Group III] = 50.72; 95% CI: 45.05 to 56.39; adjusted $p < 0.0001$) (Table 1).

Table 1: Tukey's post-hoc between-group comparisons for iNOS immunoexpression.

Comparison (first group - second group)	Mean difference	95% CI of difference	Adjusted p-value	Significance
Group I vs Group II	-60.16	-65.83 to -54.49	<0.0001	****
Group I vs Group III	-9.443	-15.11 to -3.772	0.0013	**
Group II vs Group III	50.72	45.05 to 56.39	<0.0001	****

CI, confidence interval. Mean difference is calculated as the first group minus the second group. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.3.2 Heme oxygenase gene expression statistical results:

The highest mean relative fold change in HO-1 gene expression was recorded in Group 1 (0.9890 ± 0.1293), followed by Group 3 (0.6533 ± 0.04163), while the lowest mean was observed in Group 2 (0.3100 ± 0.1015). One-way ANOVA indicated a statistically significant difference among the studied groups ($p < 0.05$).

Tukey's post-hoc pairwise comparisons showed that Group 1 had significantly higher HO-1 expression than Group 2 (mean difference [Group I - Group II] = 0.6790; 95% CI: 0.4125 to 0.9455; adjusted $p < 0.0001$) and Group 3 (mean difference [Group I - Group III] = 0.3357; 95% CI: 0.06916 to 0.6022; adjusted $p = 0.0115$). Group 3 showed significantly higher HO-1 expression than Group 2 (mean difference [Group II - Group III] = -0.3433; 95% CI: -0.6098 to -0.07683; adjusted $p = 0.0098$) **(Table 2).**

Table 2: Tukey's post-hoc between-group comparisons for HO-1 gene expression.

Comparison (first group - second group)	Mean difference	95% CI of difference	Adjusted p-value	Significance
Group I vs Group II	0.6790	0.4125 to 0.9455	<0.0001	****
Group I vs Group III	0.3357	0.06916 to 0.6022	0.0115	*
Group II vs Group III	-0.3433	-0.6098 to -0.07683	0.0098	**

CI, confidence interval. Mean difference is calculated as the first group minus the second group. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

4. Discussion:

Mono Sodium Glutamate (MSG) is a flavor enhancer with a very widespread that can stimulate sensory receptors and enhance the palatability of meals. It is found in processed meats, frozen meals, fast food, frozen dinners, potato chips, powder seasonings, and many other products. It is regularly consumed despite concerns regarding its safety.[1]

The imbalance between free radicals and body antioxidant defense can result in oxidative stress and consequently the release of ROS.[14] Camel milk has demonstrated promising potential therapeutic effects due to antioxidant properties. It has been presented in many oxidative stress disorders; consequently, it was chosen as a natural antioxidant to ameliorate the toxic effect of MSG in this study [8].

Upon the histological examination of the tongue's dorsal surface of MSG group, marked atrophy and degeneration of the surface papillae filiform, fungiform, and circumvallate were revealed. These results were analogous with the results of Fathy et al., 2019 [4] who studied the effect of MSG on albino rats' buccal mucosa and found severe histological changes and destruction to the superficial mucosa.

The filiform papillae showed areas of hyper-keratinization and other areas of keratin separation or keratolysis. This might be a protective mechanism of the body that prevented harmful food additives from penetrating the lamina propria. This might be attributed to keratin dystrophy of the covering epithelium after exposure to MSG. This finding was in alignment with Koka et al., (2022)[15] who found that it caused thickening of the tongue's covering epithelium, accompanied by hyperkeratosis. The cytoplasmic vacuolations observed among the epithelial cells might be portrayed as a cellular defense strategy against noxious substances, in addition to collecting

harmful elements and preventing their interference with surrounding cells. This went analogous with Ragab., 2018 who investigated the effect of MSG on renal tubules.[16]

The atrophy and disfigurement of the fungiform papillae in group 2, as well as degeneration of taste buds, might be caused by oxidative stress and ROS generation resulting from MSG circulation and dissociation, leading to the discharge of apoptotic factors and, subsequently, cellular damage. This is conforming to Atef et al. (2019)[17], who studied the hepatotoxic effects of MSG and found that it is hepatotoxic and has oxidant effects, as evidenced by increased lipid peroxidation and increased ROS generation.

The fibrous degeneration of the lamina propria underlying the fungiform papilla, as well as CVP, came in harmony with the outcomes of Abdelhamid et al., (2022)[18] who stated that MSG enhanced apoptosis and oxidative stress by ROS, causing degenerative changes and cellular necrosis, which might be the cause of fibrous degeneration. Controversial to these findings Abd-Elkareem et al., (2022)[19] found excessive collagen fibers production in the MSG experimental sections and related this to kidney fibrosis, where MSG intake might induce the lymphocytes to produce fibro-genic factors as lymphokines, which enhance proliferation of fibroblast, and showed as thick collagen fibers in the renal tissues. This might be attributed to differences in cellular reactions, as the tissue under investigation is different.

The degeneration of taste buds and taste cells in the CVP in this group might be attributed to glutamate excitotoxicity, which began with excess extracellular glutamate that overstimulated glutamate receptors, leading to intracellular calcium (Ca) overload. This resulted in the initiation of several events, including the activation of Ca-dependent catabolic enzymes such as phospholipases, proteases, and protein phosphatases, as well as free radical generation, mitochondrial dysfunction, and, finally, neuronal death. This was explained by Farhat et al. (2021)[20], who studied glutamate neurotoxicity on neuronal cells. Loss of salivary gland architecture was seen in serous acini of this group, along with acinar contraction and widening of CT septa matched the histological observations of Fathy et al., (2019) [5] who researched the effect of MSG on the submandibular salivary gland. This might be attributed to the oxidative stress induced by MSG, which causes lipid peroxidation, increases membrane fluidity, and inactivates membrane-bound receptors, ultimately leading to cellular disorganization.

The histological examination of group 3 (camel milk and MSG) showed improvement in the degenerative changes induced by MSG. These results were in harmony with Shaban et al., (2022) [21] who studied the impact of camel milk and its derived exosomes on diabetic nephropathy in rats that caused notable regenerative changes in renal cells. Similarly, the antioxidant effect of camel milk was also studied by Badawy et al., (2018)[22] who implied that camel milk and its derived exosomes have a potential anticancer effect when employed against induced breast cancer, possibly through inhibition of oxidative stress and inflammation.

The mean area of iNOS immunostaining in group 2 revealed a highly significant increase compared to the control group, indicating inflammation and oxidative stress. This was explained by Zhen et al., (2008)[23] who noted that iNOS expression can be triggered by oxidative stress, leading to nitric oxide (NO) generation. They also stated that overproduction of NO and ROS leads to the formation of cytotoxic compounds, causing tissue dysfunction. The immunohistochemical and statistical results for group 3 showed a reduced mean area % of iNOS immunostaining, supporting the antioxidant and anti-inflammatory properties of camel milk. This was in accordance with Badawy et al., (2018)[22] who suggested that the oxidative stress/antioxidant pathway was involved in the inhibitory potential of camel milk, and that, as a result, the iNOS oxidative stress marker was reduced.

Heme oxygenase (HO-1) is an inducible enzyme which is triggered by oxidative stress, capable of catalyzing heme degradation and inhibiting apoptosis in response to proinflammatory agonists; thus, it can minimize the deleterious inflammatory effects. HO-1 expression is upregulated under oxidative stress and hypoxic conditions. However, in the case of MSG induced hypoxia, downregulation of HO-1 expression might occur due to several underlying factors, one of which is cellular damage beyond the adaptive threshold. It was confirmed when MSG causes excessive or severe oxidative stress. [24] Camel milk is loaded with lactic acid bacteria (LAB), which is known to have high antioxidant potential. It could upregulate HO-1 gene expression, as seen in the qRT-PCR results of our study. This was consistent with Arab et al., (2021) who reported that camel milk attenuated cyclosporin-induced nephrotoxicity by augmenting antioxidant defenses, ameliorating oxidative stress and restoring HO-1 gene expression to a normal state[25].

Across all previous studies, histological, immunohistochemical, and molecular assessments confirmed that camel milk had a powerful ameliorating effect on MSG-induced toxicity on the dorsal surface of the tongue.

5. Conclusions:

The current investigation emphasized the protective role of camel milk against MSG-induced toxicity on the dorsal surface of the tongue. Owing to its strong antioxidant capacity, natural origin, and favorable safety profile, camel milk could be considered a hopeful supplement to counteract oxidative stress thereby contributing to better preservation of tissue architecture.

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