THE POTENTIAL PROTECTIVE EFFECT OF LICORICE AGAINST AGE-RELATED CHANGES IN THE CIRCUMVALLATE PAPILLAE OF ALBINO RATS

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ABSTRACT

Licorice extract has been used effectively to treat various diseases, but research on its anti-aging potential, particularly in the gustatory lingual papilla, is limited. Consequently, this study aimed to assess the potential of licorice extract to ameliorate age-related changes in the circumvallate taste papilla of rats. Thirty adult male Albino rats (6 months old) were randomly divided into three groups; Group I (adult control group) was sacrificed immediately, while Group II (aged control group) and Group III (aged licorice-treated group) were sacrificed at the age of 12 months. The circumvallate papillae in each experimental group from the tongue were analyzed histologically and histomorphometrically. The expressions of Ki-67 and neural cell adhesion molecule (NCAM) were examined using reverse transcriptase polymerase chain reaction. The superoxide dismutase levels (SOD) were determined using a colorimetric SOD activity assay. The circumvallate papillae of group II revealed various structural alterations, and its taste buds appeared shrunken and exhibited an atypical structure. While in group III, the papillae demonstrated better histological features, taste buds displayed cells with distinct boundaries; however, cytoplasmic degradation with irregular nuclei arrangement was evident in a few taste buds. Ki-67, NCAM, and SOD levels were significantly decreased in groups II & III compared to group I. However, group III exhibited a statistically significant increase compared to group II. Thus, it could be concluded that licorice extract might offer great potential as an anti-aging therapeutic compound to preserve function in the gustatory lingual papilla.

KEYWORDS: Glycyrrhiza, Neural cell adhesion molecule, Taste buds, Superoxide dismutase, Tongue

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INTRODUCTION

Aging is a natural physiological process accompanied by a gradual loss of physiological function and the degeneration of tissues and organs (1). Genes that affect metabolism, cellular senescence, cell death, DNA repair, and the antioxidant system regulate lifespan, but different organ-building tissues may exhibit various aging patterns (2). All body organs and tissues, including the soft oral tissues, are affected by aging (3).

The aging of the oral mucosa has typically been discussed in terms of changes to the oral epithelium and connective tissue. This was exhibited in specialized, masticatory, as well as lining mucosa. Clinically, the histological alterations may be accompanied by oral mucosal surfaces that are dry, thin, and smooth with a lack of elasticity and the typical stippling (4, 5). In the same context, gustatory system deterioration manifested in the form of taste loss and/or dysfunction was reported to be brought on by aging (6, 7).

Since life expectancy has increased over the past few decades, age-related disorders have become a significant issue, necessitating new treatment strategies emphasizing disease-free, healthy aging (8). Herbal medicines are sometimes referred to as the “nourishing of life” in traditional medicine, and their use as phytotherapeutics for anti-aging is becoming increasingly popular (9). The health advantages of plants are attributed to their bioactive components, which have a variety of physiological impacts on the human body (10).

Glycyrrhiza glabra L., commonly known as licorice, is a small perennial herb indigenous to Asia and the Mediterranean region. The name “glycyrrhiza” originates from the Greek words “glykos,” which means “sweet,” and “rhiza,” which means “root” (11). More than 400 bioactive molecules had been sequestered from the genus Glycyrrhiza. These compounds fall under the categories of saponins, flavonoids, chromenes, coumestans, coumarins, dihydrostilbenes, dihydrophenantherenes, and benzofurans, among which flavonoids and triterpenoid saponins are particularly prevalent in the root of licorice (12-14).

Several in vitro, animal, and clinical researches have highlighted scientific evidence related to the pharmacological characteristics of licorice, including hepatoprotective, neuroprotective, anti-inflammatory, antioxidant, anti-ulcerative, anti-carcinogenic, anti-diuretic, antibacterial, antiviral, and immunoregulatory effects (15). Moreover, the favorable effects of licorice and its constituents in preventing and treating oral diseases such as aphthous ulcers, dental caries, candidiasis, oral cancer, gingivitis, and periodontitis were reported in clinical trials (16).

Although licorice extract has been shown to be effective in treating various health problems, research about its anti-aging potential is still few, particularly in the gustatory lingual papilla. Thus, this research aimed to evaluate the potential of licorice extract to ameliorate age-related histological and molecular alterations in rat circumvallate taste papilla.

MATERIALS AND METHODS

Preparation of plant extract

Licorice-dried roots were purchased from the private market (Intenam, Egypt). The roots were powdered and extracted with 70% ethanol using an ultrasound-assisted extraction method. After that, the extract was filtered and concentrated under reduced pressure. The extract was then dissolved in normal saline to reach the desired concentration (2mg/ml) and kept at 4°C for further use.

Experimental animals

The experiment was conducted at the animal house of the Faculty of Medicine, Cairo University, in compliance with guidelines approved by Institutional Animal Care and Use Committee.
(CU-IACUC), Cairo University (approval number CU III F C 64 21). Thirty adult male Albino rats (6-months-old) with an average weight of about 160-200 g were purchased and employed in the study. The rats were obtained from the animal house of the Faculty of Medicine, Cairo University. They were housed in individual stainless-steel cages under a controlled environment (temperature 24 ± 1 °C, relative humidity 45 ± 10 %, and 12-hour dark/light cycles). The rats were enabled for free to get their basic diet of regular rat chow (Ibex International, Giza, Egypt) and tap water ad libium throughout the whole experimental period.

Experimental design

The 6-months-old rats were randomly divided into 3 groups (n=10/group) as follows:

**Group I: Adult control group.**

The rats were sacrificed immediately at the beginning of the experiment.

**Group II: Aged control group.**

The rats received 12.4 mg/kg normal saline once daily via oral gavage for 6 months until the age of 12 months; then, they were sacrificed.

**Group III: Aged licorice-treated group.**

The rats received 12.4 mg/kg licorice extract once daily via oral gavage for 6 months until the age of 12 months; then, they were sacrificed.

Euthanasia for all the experimental animals was performed by injection of sodium pentobarbital overdose (100 mg/kg) intraperitoneally (17). The circumvallate papillae were carefully dissected in each experimental group from the tongue. Specimens for histological examination were immediately fixed in a 10% neutral buffered formalin solution. At the same time, those for quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme activity assay were immediately frozen in liquid nitrogen and stored at -80 °C until use.

Histological examination

10% neutral buffered formalin solution was used to fix circumvallate papilla tissues for 48 hours. After being dehydrated in ascending grades of ethanol, the tissues were cleared in xylene and then embedded into paraffin. The paraffin-embedded specimens were sectioned at 5μm thickness and then placed on a glass slide for hematoxylin and eosin (H&E) staining (18). Under a light microscope (Leica DM 1000), stained sections were examined for overall histological changes.

Histomorphometric analysis

Images for each specimen from the three experimental groups were collected by light microscope using an objective lens of magnification (x200). Measurement of epithelial thickness as well as counting the number of taste buds were done using Image J analysis software (version 1.53d; NIH, Bethesda, MD, USA).

Quantitative real-time PCR examination

Total RNA was isolated from the epithelium of circumvallate papilla using Qiagen tissue extraction kit (Qiagen, USA) following the manufacturer’s instructions. The purified total RNA was then subjected to reverse transcription into single-strand cDNA with a reverse transcription kit (Fermentas, USA). qRT-PCR was performed on a Step One thermocycler (Applied Biosystems, Foster City, USA) using SYBR Green/ROX qPCR Master Mix (Thermo Fisher, USA). The sequences of primers for Ki-67 and neural cell adhesion molecule (NCAM) genes were displayed in Table 1. The used housekeeping gene was Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The ΔΔCt method calculations was used to obtain relative gene expression levels (19).

Antioxidant enzyme activity assessment

According to the manufacturer’s instructions, superoxide dismutase (SOD) levels were determined using a colorimetric SOD activity assay kit (ab65354, Abcam).
TABLE (1) Primers used for quantitative real time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Gene bank accession number</th>
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| NCAM  | F: 5′-GGTTCGAGATGGTCAGTTGCT-3′  
R: 5′-CAAGGACTCTCGTTCACCAATCCGG-3′ | NC_000011.10 |
| Ki-67 | F: 5′-TCTGATGTTAGGTGTTTGAG-3′  
R: 5′-CACTTTTCTGGTACTTCTTG-3′ | NM_001081117 |
| GAPDH| F: 5′-ACAGTCCATGCCATCACTGCC-3′  
R: 5′-GCCTGCTTCACCACCTTTGC-3′ | NG_009348.3 |

Statistical analysis

The data were statistically analyzed using the statistical package for the Social Sciences (SPSS) version 25 (IBM Corp., Armonk, NY, USA). All the data obtained from histomorphometric, qRT-PCR and enzyme activity examination was described in terms of mean and standard deviation (SD). A one-way ANOVA test was used to compare the different experimental groups. Tukey’s post hoc test was followed for multiple pairwise comparisons. A p-value less than 0.05 was considered statistically significant.

RESULTS

Histological results

Histological examination of samples from adult control rats (6-months-old) (group I) exhibited normal inverted cone-shaped circumvallate papilla surrounded by a deep narrow trough. The papilla consisted of keratinized stratified squamous epithelium overlying a connective tissue core (lamina propria). The epithelium showed properly arranged and well-defined epithelial cell layers. Numerous prominent regularly arranged epithelial ridges were demonstrated interdigitating with well-defined connective tissue papillary layer. Laterally, the epithelium lining the trough harbored numerous barrel-shaped taste buds consisting of spindle-shaped cells with prominent nuclei. Most taste buds were seen extending from the basement membrane to the trough’s surface. The lamina propria showed well-organized, dense fibrous arrangements (Figure 1 A-D).

Examination of the circumvallate papillae of aged control rats (12-months-old) (group II) revealed various structural alterations as compared to adult control rats (6-months-old). Thinning of the overlying covering epithelium was observed with a marked diminish in granular and spinous cell layer thickness. The epithelial ridges showed irregular arrangement as well as variation in length. Areas of degeneration were demonstrated in the epithelium lining the trough. The taste buds appeared shrunken and exhibited an atypical structure. Some taste buds showed severe structural deterioration. Most of the taste buds’ cells revealed loss of their boundaries, degradation in cytoplasm, dearranged nuclei, and some demonstrated complete nuclei loss. Distorted lamina propria with dissociated, loose disorganized fibers were observed. Marked inflammatory cellular infiltration was also evident (Figure 1 E-H).

Unlike the aged control rats, the circumvallate papillae of aged licorice-treated rats (group III) revealed better histological features. The overlying epithelium was noticeably thicker with proper cellular layer arrangement except for disruption in basal and parabasal cell arrangement that was displayed in some minute areas. Moreover, focal areas of decreased epithelial cell density were observed. The epithelial ridges appeared regularly arranged. Relatively well-defined barrel-shaped taste buds were observed in the lateral lining epithelium facing the trough. Most taste buds displayed cells with distinct boundaries and prominent nuclei; however, cytoplasmic degradation with irregular nuclei arrangement was evident in a few taste buds. The underlying lamina propria appeared denser and more organized with fewer fibrillar dissociation areas than the aged control group (Figure 1I-L).
Histomorphometric results

**Epithelial thickness**

Statistical analysis showed significant difference in mean epithelial thickness among all experimental groups using ANOVA ($p<.0001$). The mean epithelial thickness was statistically significantly decreased in aged control rats (group II) ($p<.0001$) and non-significantly decreased in aged licorice-treated rats (group III) ($p=.1839$) as compared to adult control (group I) ($p<.0001$). On the other hand, the mean thickness was significantly increased in aged licorice-treated rats as compared to aged control rats ($p<.0001$) (Figure 2A).

**Taste buds number**

ANOVA test demonstrated statistical significant difference in mean taste buds number between all experimental groups ($p=.0106$). Statistically significant decrease in mean taste buds number was demonstrated in aged control rats versus adult control ($p=.0076$). However, this decrease in the aged licorice-treated rats in relation to adult control was statistically non-significant ($p=.2201$). In comparison to aged control group, the mean taste buds number was increased in aged licorice-treated group however it was statistically non-significant ($p=.2723$) (Figure 2B).

**Gene expression results**

The relative gene expression of Ki-67 and NCAM demonstrated a significant difference among all experimental groups using ANOVA ($p<.0001$). Statistically significant decrease in the expression levels of Ki-67 and NCAM was demonstrated in aged control rats ($p<.0001$) as well as aged licorice-treated rats ($p=.0088$ & $p=.0066$ respectively), compared to adult control rats. However, compared
to aged control rats, aged licorice-treated rats exhibited a statistically significant increase in Ki-67 and NCAM gene expression (\( p = .0006 \) & \( p = .0007 \), respectively) (Figure 3).

**SOD enzyme activity**

Using ANOVA, SOD levels significantly differed across all experimental groups (\( p < .0001 \)). Compared with the adult control rats, the SOD levels were statistically significantly decreased in aged control rats (\( p < .0001 \)) and licorice-treated rats (\( p = .0228 \)). On the other hand, statistically, the SOD level was increased significantly in the aged licorice-treated group relative to the aged control group (\( p = .0038 \)) (Figure 4).
DISCUSSION

Aging impairs both immunologically and physically the mucous membrane’s defensive mechanisms (3). The gustatory systems’ functionality also tends to decline with age (20-22), affecting one’s general health, and quality of life. Medicinal plants and their active ingredients are a great source of knowledge for developing novel therapies to improve human health and lifespan. This study explored the potentiality of licorice extract in attenuating structural changes associated with aging in circumvallate taste papilla of male Albino rats. As far as we know, this research is the first to demonstrate that licorice extract has a beneficial anti-aging impact in a gustatory lingual papilla model.

The circumvallate papilla represents a key compartment of the gustatory system in the tongue as it is the home to more than 500 taste bud apertures (23,24). Thus, in this study, it served as a good candidate model for detecting structural alterations being researched in the mucosa as well as taste buds.

In the present work, young adult rats 6-month-old received oral licorice extract daily for 6 months at a dose of 12.4 mg/kg. This dose was chosen based on earlier research found in the literature, where the same dose of licorice extract given to ovariectomized rats for 6 months did not induce any clinically harmful effects. This dose was therefore determined to be safe and non-toxic after prolonged administration (25).

Histologically, regressive changes in the epithelium and the underlying lamina propria of the circumvallate papilla were displayed in aged control rats (group II) compared to adult control rats (group I). Our results demonstrated diminish in the epithelial layer, denoting progressive epithelial atrophy in aged rats. Histomorphometrically, this decrease was statistically significant. A similar finding was previously reported by Elias (26), who found that the buccal mucosa of 1-year-old rats had less epithelial thickness and that the cells of different layers appeared diminished and reduced in height than those of 6-month-old rats. Moreover, several other reported age-related cellular alterations in oral mucosa aligned with our findings. These included decreased cellular density, and altered junctions between the epithelium and connective tissue (5, 27).

In our study, the aged rats’ lamina propria displayed dissociated, loose disorganized fibers. This was consistent with Lamster et al. (28), who found that, with aging, the oral mucosa demonstrated a loss of elastic fibers and disorganization of collagen bundles in the connective tissue. In the same context, increased connective tissue proteolysis was reported in aged human skin. This was attributed to the increase in collagenases expression and the decrease in collagenases inhibitors (29). On the contrary, another study by Kang et al. (30) showed increased collagen and connective tissue deposition in the rat’s gastric mucosa with aging. These contradicting reports might be related to the different compositions of proteoglycans and collagen types of connective tissue in various organs leading to variations in the connective tissue aging process (31,32).

In aged licorice-treated rats, we observed marked attenuation in the age-related histological changes of the circumvallate papilla, where the epithelium and the underlying lamina propria partially preserved much of their normal histology. Similarly, Kong et al. (33) found that licorice extract significantly reduced histopathological damage in a mouse model of UV-induced skin photoaging.

In this study, concomitant with our histological findings, we found a significant decrease in gene expression levels of the cell proliferation marker Ki-67 in aged control relative to adult control rats. This finding was in agreement with other researches (26, 34, 35) that demonstrated a significant decrease in epithelial proliferation and rate of tissue turnover with age. Our results also revealed that aged rats treated with licorice showed significant increase in Ki-67 expression compared to those untreated. This denoted that licorice treatment attenuated the
decline in cell proliferation encountered during aging.

The tendency of licorice treatment to stimulate cell proliferative activity was previously observed in different *in vitro* (36,37), animal (38,39) as well as clinical models (40). In contrast, it’s interesting to notice that licorice had been shown to cause apoptosis and cell cycle arrest in various cancer cell line types, exerting an anticancer impact (41,42). This combination of opposing effects indicate that licorice treatment interacts differently with healthy normal cells than it does with cancer cells.

In the current work, the taste buds of the circumvallate papilla in the aged rats showed structural atrophy as well as significant decrease in their number compared to adult control rats’ Our findings supported earlier research by Fukunaga et al. (43), who found that aged mice have slower cell renewal and heavily vacuolated cytoplasm in their taste buds than young adult mice. Furthermore, in mice circumvallate papillae, Shin et al. (22) demonstrated a significant age-related decline in taste bud size, number of taste cells per bud, and taste cell markers.

Unlike the aged control group, most of the taste buds in the aged licorice-treated group in the present study displayed structural atrophy as well as prominent nuclei. These findings suggested that licorice treatment preserved to a considerable degree, the structure of taste buds histologically during the normal aging process.

Mammals have four types of taste cells in their taste buds (types I, II, III, and IV), each with a unique molecular phenotype and functional purpose. The ability to form classical vesicular synaptic contacts with gustatory nerve terminals is one of the key features of type III taste cells (44). NCAM is a membrane-surface glycoprotein that is present in neural tissues involved in cell-cell interactions such as adhesion and recognition (45). It has been shown to play a role during synaptogenesis, recruiting and stabilizing the vesicular pool, in addition to its function in synapses adhesion (46,47). Both type III cells and nerve fibers in taste buds express NCAM (48). In this regard, NCAM is considered a reliable neurological marker.

Consistent with earlier published results (49,50), our qRT-PCR analysis revealed a significant decrease in NCAM gene expression in aged rats compared to adult rats. Our findings suggest that the decline in NCAM expression in aged rats might disrupt NCAM-mediated functions particularly synaptic functions. Consequently, the gustatory function might be compromised. Our hypothesis corroborated prior research that suggested aging might alter taste sensitivity (21, 51).

In accordance with the reported positive therapeutic effect of licorice observed in the present study, the NCAM gene expression was significantly increased in aged licorice-treated rats in relation to untreated aged ones. This could reflect proper maintenance of gustatory neural activity in aged rats treated with licorice. In agreement with this, several aging models have previously shown that licorice extract and its isolated biological components had neuroprotective effects (52-54).

According to damage theories, one of the key factors in the aging process is the buildup of reactive oxygen species (ROS), which non-specifically oxidize cellular components such as proteins, nucleic acids, and lipids. This causes an accumulation of oxidative damage in organisms, contributing to age-related illnesses and pathologies (55, 56). SOD catalyzes the degradation of superoxide radicals to hydrogen peroxide and oxygen; hence, it is the primary enzymatic scavenger of ROS. It is well-established that SOD is one of the most effective mechanisms in the antioxidant system (57).

The current investigation demonstrated a significant reduction in SOD levels of aged rats compared with adult rats. This decrease in SOD levels suggests that the antioxidant system has become compromised with age, resulting in an inability to protect cells from oxidative stress.
result, aged tissues experience marked histological and morphological alterations (58). In the same context, it was found that higher levels of free radicals result in lipid peroxidation of neural cell membranes (59), a reduction in full-length NCAM-180 (60), destruction of the protein Ki-67 (61), and a reduction in cell proliferation (62). In this respect, the pronounced histological changes as well as the decline in neural and proliferation-related gene expression observed in this study in the aged rats, might be significantly linked to the considerable decline in the antioxidant parameter SOD.

Notably, the SOD levels were significantly increased in aged licorice-treated rats versus aged untreated rats in this work. This finding supported the licorice treatment’s reported ability to reduce oxidative stress in several earlier experimental models (53, 63, 64). We hypothesized that licorice’s antioxidant capability played a significant role in its therapeutic benefits against age-related alterations observed in the circumvallate papilla in this study.

The biological benefits of licorice have been reported to lie in its chemical constituents. The powerful antioxidant potential of licorice was significantly linked to its content of flavonoids (65). Several flavonoid compounds have been identified from licorice, including flavanones, isoflavonones, flavones, isoflavones, flavonols, isoflavenes, isoflavans and chalcones (66). The antioxidant capacity of flavonoids was shown to be related to its molecular structure. Most reported isoflavonoids are characterized by a prenyl moiety on rings A or B. This isoprenyl group enhances the lipophilicity of flavonoids backbone, increasing its affinity for cellular membrane structures and favoring positive biological activities (67).

Licorice content of licochalcone A, licoisoflavone, and isolicocflavonol was reported to reduce paraquat-induced oxidative stress in lung tissues through increasing the SOD activity and decreasing malondialdehyde levels (68). Glabridin, one of Glycyrrhiza glabra’s isoflavone derivatives, was also reported to protect mitochondrial functions from oxidative stresses by preventing microsomal lipid peroxidation (69).

In addition to its antioxidant activity, licorice phytoconstituents may also function in other ways that may have contributed to the positive therapeutic effects observed in the current study. Glycyrrhizin (triterpenoid saponins) had a mitogenic impact through epidermal growth factor receptors, stimulating the mitogen-activated protein kinase pathway to induce DNA synthesis and cell proliferation (70). It was also found that glycyrrhizin delayed tissue aging and encouraged cell regeneration by suppressing senescence-associated secretory phenotype indicators (54, 71, 72).

Furthermore, glycyrrhetic acid was found to act on the level of the presynaptic membrane, where it enhanced spontaneous secretion of transmitter from neurotermination of the neuromuscular synapses in a dose-dependent manner (73). Isoliquiritigenin, was also observed to ameliorate synaptic dysfunction and promote synaptic plasticity through increased protein levels of synaptophysin (74) as well as increased expression of brain-derived neurotrophic factor (75).

Based on these reports, it could be assumed that the bioactivity of licorice extract is not contributed to individual phytoconstuent but to the overall phytochemical composition. Finally, the herein reported results from different investigatory tools, denoted that licorice treatment slowed down the normal physiological aging process to a great extent, but it couldn’t prevent it.

**CONCLUSION**

In conclusion, the results of the current work indicated age-related alterations in rat circumvallate taste papilla. These changes might lead to deterioration in somatosensory responses to tastants. Licorice extract effectively attenuated these changes by increasing the activity of SOD enzyme to inhibit oxidative stress and increased gene expression of Ki-67 and NCAM. As a result, licorice extract might
offer great potential as an anti-aging therapeutic compound to preserve function in the gustatory lingual papilla. More thorough in vivo preclinical investigations are required to evaluate the dose and the safety of licorice compounds when used for extended periods. Likewise, due to the complex interaction between the herb’s multiple bioactive components and targets in the organisms, more studies are needed to find more about the molecular mechanisms underlying the pharmacological action of licorice.

Conflict of interests

There are no conflicts of interest.

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