Age-related changes of the tongue and Weber's salivary glands in male albino mice: A histopathological and morphometric study

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SUMMARY

The tongue and salivary glands are affected by aging process. This study aimed to assess the age-related structural changes of the tongue and Weber's salivary glands in mice. Mice were grouped as adult (5.9 ± 0.5 months old) and aged (21.2 ± 0.6 months old) groups. After sacrifice, the tongues in both groups were dissected and underwent a histopathological examination using haematoxylin and eosin (Hx&E) stain, as well as morphometric analysis. The aged group exhibited thin epithelium, atrophied papillae, engorged blood vessels, disorganized myofibers and increased adipose tissue deposition. In addition, the lingual salivary gland revealed acinar cell atrophy and increased interacinar stroma. The lingual lymphoid tissue showed diffusely arranged lymphoid cells and increased stroma. Morphometrically, the aged group exhibited a significant decrease in the height and number of filiform papillae, in addition to a significant decrease in epithelial layer thickness, diameter and length of myofibers, length of myonuclei, and the number of sarcomeres. Furthermore, the aged group showed a significant increase in lamina propria/submucosal thickness, length of sarcomeres and stromal tissue percentage of the Weber's salivary glands. Aging induces significant structural changes in the tongue and Weber's salivary glands in mice.

Key words: Aging – Tongue – Salivary gland – Histopathology – Morphometry

INTRODUCTION

Feeding is considered as a critical factor that ensures the success for adaptation to the environment and the persistence through procreation (Iwasaki, 2002). The tongue is considered a mirror reflecting general personal health, particularly the filiform papillae (Altayeb and Salem, 2017). Structurally, it is a muscular organ enclosed by epithelium, and contributes to taste, speech, swallowing, suckling and respiratory functions (Byahatti et al., 2010; Nagai et al., 2008; Patil et al., 2013). The papillae on the dorsal surface of the tongue are divided into 2 types; mechanical papillae and gustatory papillae (Goździewska-Harłajczuk et al., 2018). Moreover,

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the taste buds are widely distributed over its dorsal and lateral surfaces indicating the critical importance of the tongue in feeding and taste (Iwasaki, 2002).

The aging process is implicated in several anatomical and functional changes (Junior et al., 2014). One of the largely affected organs by these aging changes is the tongue because it continues to grow even at advanced age affecting both taste acuity and structure (Rother, et al., 2002; Conger and Wells, 1969). For example, by the age of 75-90 years old, it was reported that the number of taste buds is 50% less than that in younger individuals (Mistretta, 1984). Furthermore, there is a progressive decline in skeletal muscle mass and strength with age (Cheng et al., 2017). Even histologically, as noted in human cadaveric tongues, with progressive aging there is a corresponding reduction in tongue muscle fiber diameter of the superior longitudinal muscle fibers (Nakayama, 1991).

Age-related lingual dysfunction may largely affect swallowing due to muscle fibers affection and an increased noncontractile tissue, causing a stiffer tongue; in addition, aging could also increase the risk of aspiration, as it increases the probability of bolus retention in the pharynx (Connor et al., 2009; Cichero, 2018; Yoshida et al., 2006). Furthermore, age-related taste disorder consequences may take place such as anorexia, loss of appetite, changes in food preferences, weight loss and malnutrition (Imoscopi et al., 2012). These impaired functions may finally cause considerable morbidity and mortality through increased risk of starvation, dehydration, aspiration pneumonia and airway obstruction (Palmer et al., 2000).

So, the aim of this study was to investigate the age-related histopathological and morphometric changes of the tongue and Weber's salivary glands by comparing aged and adult mice.

MATERIAL AND METHODS

Animals

Twenty pathogen-free Swiss-albino male mice were purchased from the Animal House of the Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt. All experimental procedures were performed regarding the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

Experimental design

The obtained mice were selected according to their ages to be divided into 2 age groups (n=10): adult group (5.9 ± 0.5 months old) and aged group (21.2 ± 0.6 months old). These age groups were chosen to be equivalent to human ages of about 20 years old for the adult group and about 70 years old for the aged group. This estimation of age in mice in comparison to the human was calculated according to the following equation:

9.125 mice days = 1 human year (Dutta and Sengupta, 2016).

Histopathological study

All animals were euthanized by an overdose of intraperitoneal sodium pentobarbital. The tongue was carefully dissected out and submersed in 10% formal saline for not more than 6 hours. Specimens were then dehydrated, cleared in xylene and embedded in paraffin. Sections of 5 µm thickness were cut at the mid-sagittal plane of the tongue with vertical cuts, stained with haematoxylin and eosin (Hx&E) stain and then examined by light microscope (Olympus, Tokyo, Japan). The dorsal aspect of the anterior part of the tongue was examined for mucosal and muscle layers, while its posterior part was examined for its lymphoid tissue and Weber's salivary glands.

Morphometric study

Morphometric measurements were performed on the original images using the ImageJ® (version 1.53) software. To avoid the inter-observer bias, only one investigator performed the morphometric measurements. From each animal, 5 sections were observed and from each section 5 different regions were investigated. In a pre-elementary pilot study, we found that formalin fixation for not more than 6 hours of tongue specimens resulted in 6.4 ± 0.3% tissue shrinkage, so we considered

this observation during our measurements and the statistical analysis. The following parameters were measured in Hx&E-stained sections regarding mucosal layer at a magnification of ×400:

1. Total epithelial thickness: the distance between the basal lamina of the epithelial layer to the surface of horny layer.
2. Horny layer thickness: the distance between the end of granular layer to the tips of filiform papillae.
3. Total height of filiform papillae: the distance between the base to the tip of filiform papillae.
4. Lamina propria/submucosa thickness: the distance between the basal lamina of the epithelial layer and the muscle layer.
5. The number of papillae/mm.
6. Percentage of stromal tissue of Weber's salivary glands: the percentage of the stromal tissue surface area in relation to the total surface area of the examined image.
7. Proportions of total epithelial, horny layer and lamina propria/submucosal thicknesses according to the total thickness of the tongue were calculated. The total thickness of the tongue was measured at median sagittal Hx&E-stained sections at a magnification of ×40 by measuring the distance between a horizontal line passing through the midway between dorsal and ventral surfaces, and the dorsal surface exactly at the middle of it to avoid duplication between the layers of the dorsal and ventral sides during the calculation of these proportions. The middle point of the dorsal surface was determined by measuring the total length of it from the tip of the tongue to its root, so the midpoint was detected (Fig. 1).

Furthermore, the following measurements were applied regarding the muscle layer at a magnification of ×1000:

1. Diameter of myofibers.

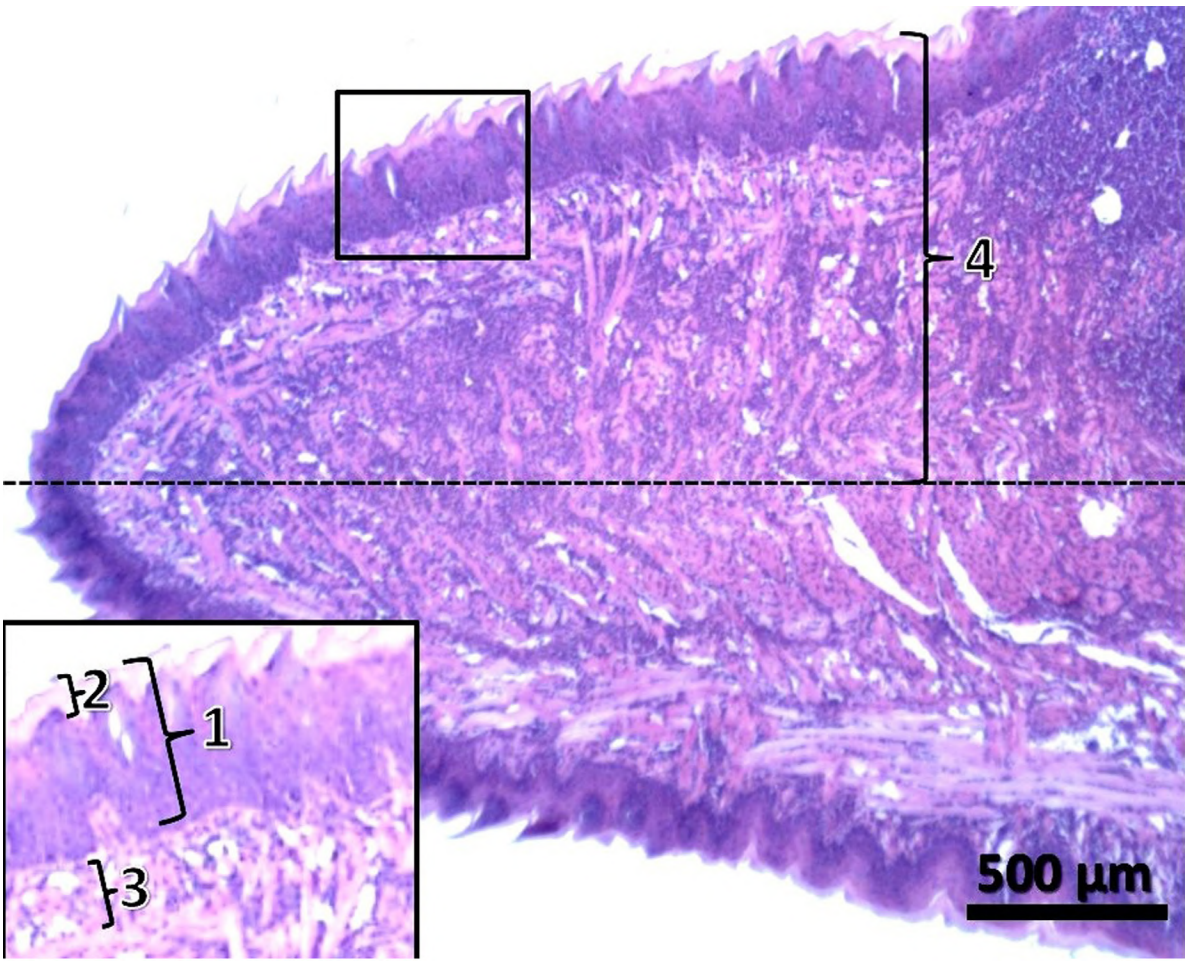

Fig. 1.- Procedures for measurement of proportions of the total epithelial (1), horny layer (2) and lamina propria/submucosal (3) thicknesses according to total tongue thickness (4). (Hx&E; ×40 – scale bar = 500 µm).

2. Length of myonuclei.
3. Length of sarcomeres (Vidal et al., 2020): the distance between two successive Z-lines. At high magnification, this Z-line appeared in Hx&E-stained sections as a dark line extending in the middle of the lightly stained I-band (Fig. 2).
4. Number of sarcomeres/mm (Vidal et al., 2020): each sarcomere is the area extending between two successive Z-line (Fig. 2). The number of sarcomeres was counted within the total width of the image then this counted number was divided on the total width of the image that was 0.958 mm.

Statistical analysis

The data analysis was performed using Statistical Package for Social Sciences® (SPSS) software version 25.0 for Windows. Results were expressed as the mean±standard deviation (SD). Data underwent testing for normality by Shapiro-Wilk test and revealed that the data has non-normal (non-parametric) distribution. Thus, for comparisons between the two groups, significance was evaluated using the Mann-Whitney non-parametric U-test.

RESULTS

Histopathological study

The Hx&E-stained sections of the adult group exhibited that the dorsal aspect of the anterior part of the tongue was covered by keratinized stratified squamous epithelium showing lingual papillae mostly of filiform type. The subepithelial layer of loose connective tissue was seen separating the overlying epithelial layer from the underlying muscle layer. The muscle layer showed well-arranged myofibers of different directions with distinct striations and peripheral myonuclei (Figs. 3A, B). On the other hand, the posterior part of the tongue showed embedded Weber's salivary

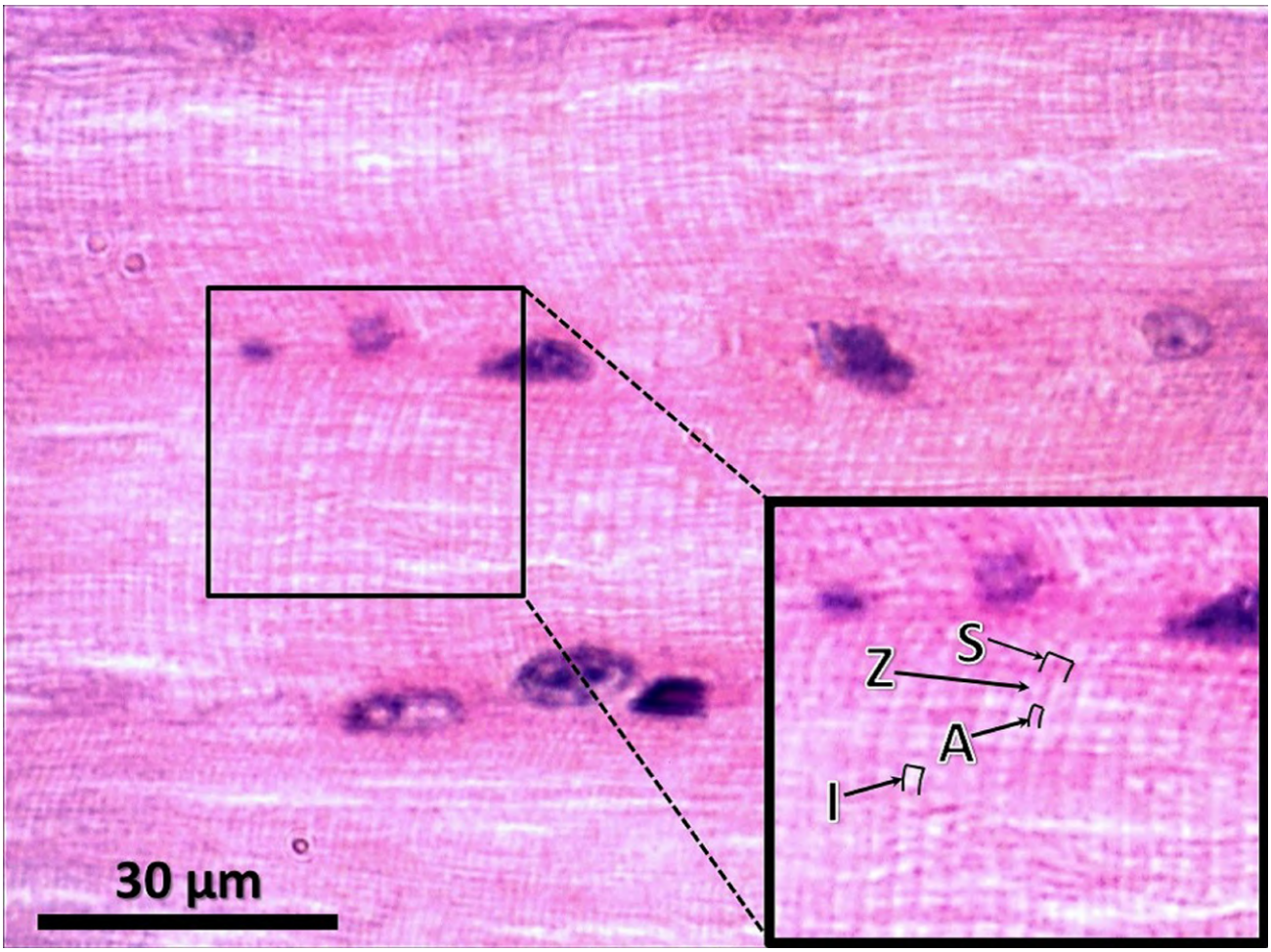

Fig. 2.- Structure of a tongue muscle fiber at high magnification showing structures used in morphometry including Z-line (Z), sarcomere (S), A-band (A) and I-band (I). (Hx&E; ×1000 – scale bar = 30 µm).

glands within the submucosa and muscle layer with purely mucous acini having a clear basophilic cytoplasm with scanty stroma in between (Fig. 3C). Furthermore, the posterior part of the tongue exhibited closely packed lymphoid cell aggregates with little stroma in between and surrounded by a distinct fibrous capsule (Fig. 3D).

The epithelial layer in the aged group revealed massive thinning of the covering keratin and areas of absent papillae. Furthermore, the underlying connective tissue was massively condensed with highly congested blood vessels (Fig. 4A). On the other hand, the muscle layer showed massive disarrangement of its myofibers, loss of most of their striations, and massive deposition of adipocytes in between muscle fibers (Figs. 4B, C). Moreover, many acini of Weber's salivary glands demonstrated loss of their epithelium and an increase in the amount of interacinar stroma (Fig. 4D). In addition, the lymphoid aggregates in the posterior part of the tongue had loosely arranged lymphoid cells with an increase in the amount of the stroma in between and thickening of the surrounding capsule (Fig. 4E).

Morphometric assessment

The total epithelial and horny layer thicknesses and proportions were significantly decreased in the aged group compared to the adult one, while the lamina propria/submucosa thickness and proportion, and percentage of the stroma of Weber's salivary glands were significantly increased in the aged group compared to the adult one (Table 1). On the other hand, both total

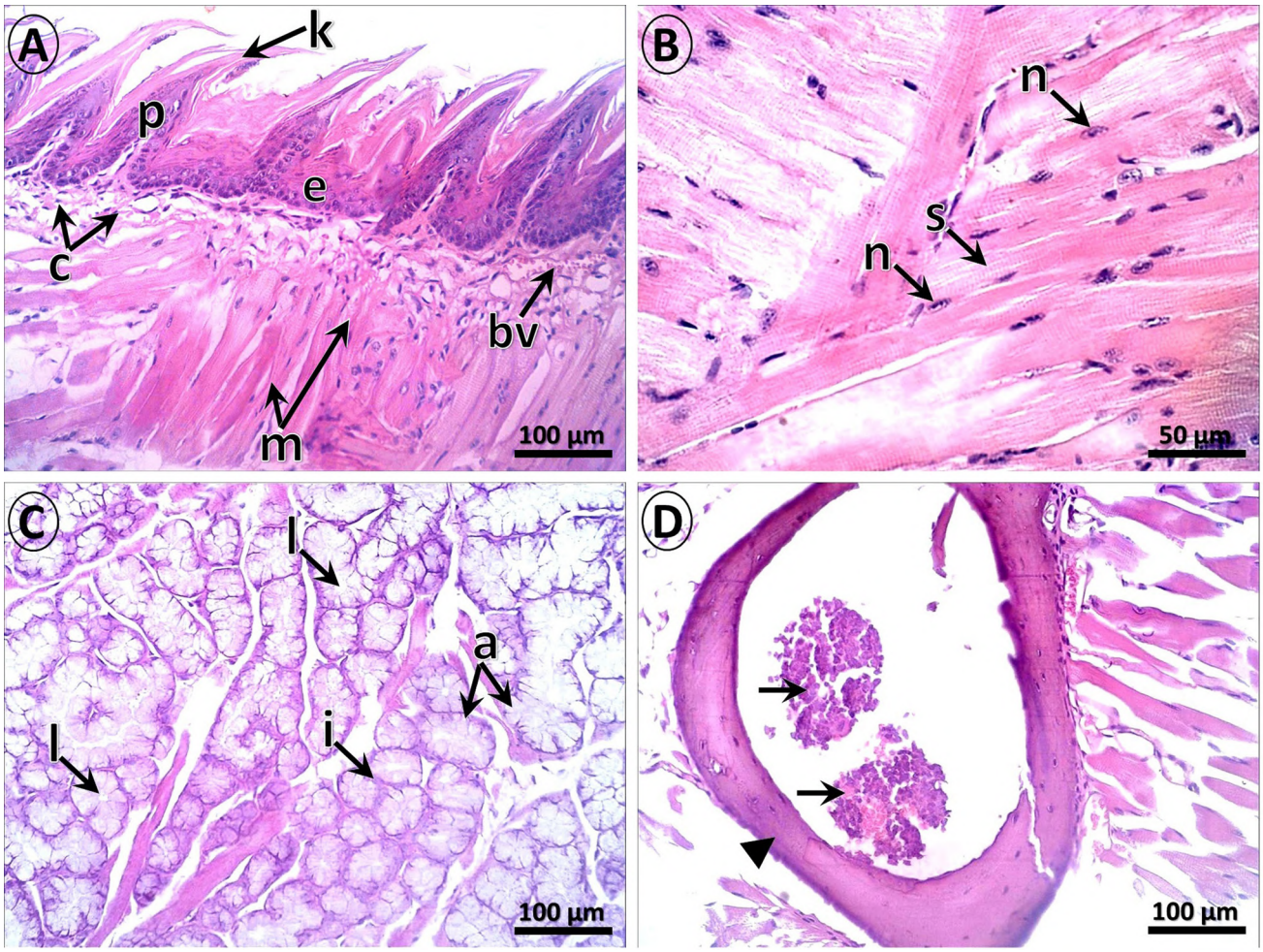

Fig. 3.- Sections of the tongue and Weber's salivary glands of the adult group. **A.** The dorsum of anterior part of the tongue showing a covering layer of keratinized stratified squamous epithelium (e) with filiform lingual papillae (p) that are covered by a keratin layer (k). There is a subepithelial layer of loose connective tissue (c) showing normally appearing blood vessel (bv), in addition to the muscle layer (m). **B.** The muscle layer of the tongue shows well-arranged muscle bundles of different directions with distinct striations (s) and peripheral myonuclei (n). **C.** The posterior part of the tongue shows Weber's salivary glands within the muscle layer with relatively large mucous acinar cells having a clear basophilic cytoplasm, central lumens (l) and scanty interacinar stroma (i). **D.** The posterior part of the tongue shows packed lymphoid cell aggregates with little amount of stroma in between (arrows), surrounded by a fibrous capsule (arrowhead). (Hx&E; A, C, D and E ×200 – scale bar = 100µm; B ×400 – scale bar = 50µm).

height of filiform papillae and their number were significantly decreased in the aged group compared to the adult one (Fig. 5). According to muscle layer morphometry, the diameter and length of myofibers, the number of sarcomeres, and length of myonuclei were significantly decreased in the aged group compared to the adult one. However, the length of sarcomeres was significantly increased in the aged group compared to the adult one (Table 2).

DISCUSSION

The tongue has a critical role in swallowing, speech and breathing (Kletzien et al., 2018). Since aging disrupts the body functions leading to increased morbidity, the tongue is one of the seriously affected organs with these aging changes manifested mainly by depapillization, in addition to reduced saliva production that makes the oral mucosa massively inelastic and dry (Kletzien et al., 2018; Warraich et al., 2020). In addition to surface fissuring and taste disturbances, as a common effect of aging on the tongue, the lingual muscles show loss of their usual tone (Malik et al., 2015; Zagaria, 2019). The current study assessed age-related changes in tongue mucosa, muscles, and lymphoid tissue, and also those involving the Weber's salivary glands in mice based on histopathological and morphometric examinations.

Experimental non-human models are essential in studying the aging process (Folgueras et al., 2018). For that, mice are considered extremely similar to humans in many physiologies and cellular functions. For example, the digestive, immune, musculoskeletal and endocrine systems of mice are quite equivalent to that of humans in both function and structure (Vanhooren and Libert, 2013). Mice are therefore a unique tool for the assessment of aging changes. In addition, the fact that mice have a short life span is an advantage, so mouse histological alterations can be examined at all stages of the aging (Nadon, 2004; Folgueras et al., 2018).

However limited, previous studies have experimentally investigated tongue age-related structural and histomorphometic alterations (Stablein and Meyer, 1986; Meisel et al., 1987). For example, and in agreement with our results,

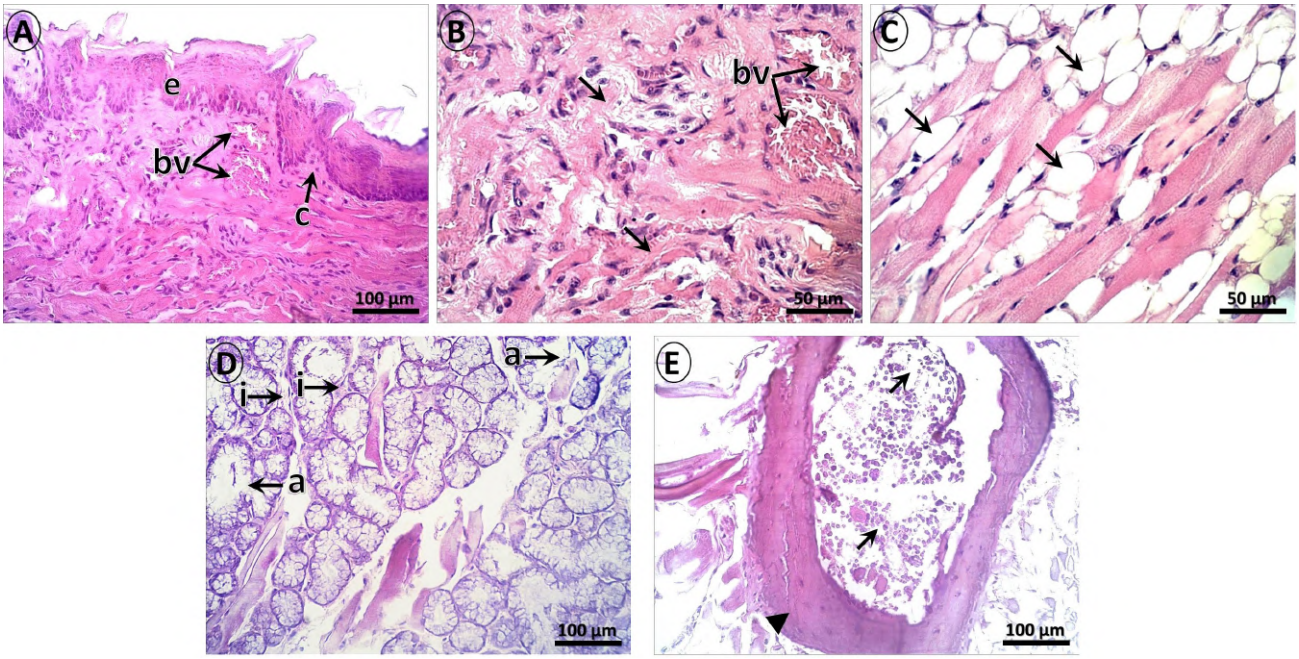

Fig. 4.- Photomicrographs of sections of the tongue and Weber's salivary glands of the aged group. **A.** The dorsum of anterior part of the tongue shows thinning of the epithelial layer (e), in addition to the absence of most the papillae. The underlying connective tissue is highly condensed (c) with massive congestion of the blood vessels (bv). **B.** The muscle layer of the tongue shows massively disorganized myofibers with loss of the boundaries between them (arrows). Note the highly congested blood vessels (bv). **C.** The muscle layer of the tongue shows infiltration with adipocytes in between its muscle fibers (arrows). **D.** The posterior part of the tongue shows Weber's salivary glands revealing degeneration of many acinar cells with loss of cell boundaries in between (a), in addition to an increase in amount of interacinar stroma (i). **E.** The posterior part of the tongue showing massively diffuse arranged lymphoid cells with increased stroma (arrows) and thickened surrounding capsule (arrowhead). (Hx&E; A, D and E ×200 – scale bar = 100µm; B and C ×400 – scale bar = 50µm).

Meisel et al. (1987) reported that aging causes various lingual mucosal changes in 18 month-old mice in form of atrophic hyperkeratotic changes of lingual epithelium with blunt disorganized filiform papillae. Nonetheless, most of the studies were concerned with the assessment of human age-related tongue changes (Scott et al., 1983; Bässler, 1987; Nakayama, 1991; Sasaki, 2004). Scott et al. (1983) found that lingual epithelial thickness in humans undergoes reduction by about 30% comparing the ages of 18 with 98 years old. Also, the current study results revealed that there is a reduction in total epithelial thickness by 38.69% in the aged mice compared to the adult ones. Furthermore, Nakayama (1991) found that the aged human tongue undergoes progressive epithelial layer degeneration, atrophy of the acini of the Weber's salivary glands and decreased

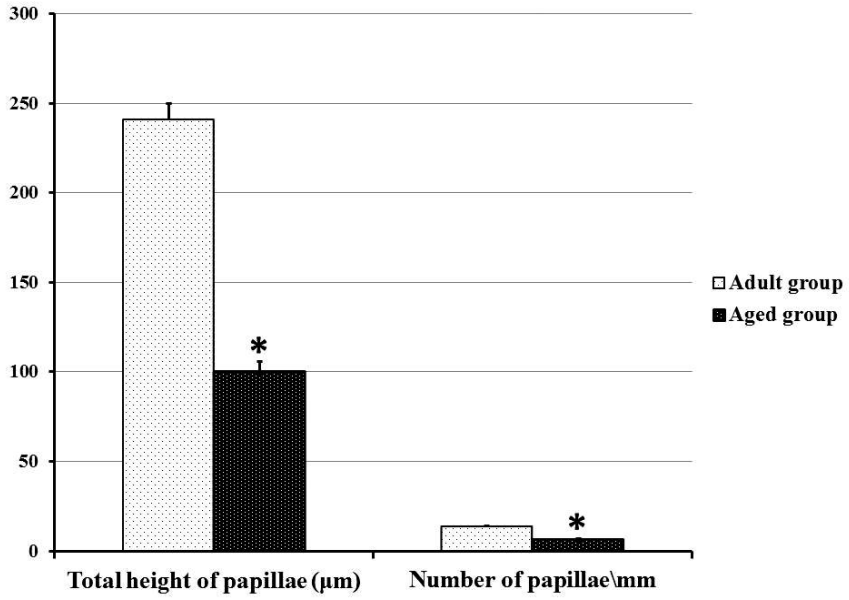

Fig. 5.- Morphometric parameters of filiform papillae of the study groups according to total hight and number of papillae. Values are mean±SD. *p<0.05 *vs* adult group, as determined by Mann-Whitney *U*-test.

Table 1. Morphometric parameters of lingual mucosa and Weber's salivary glands of the study groups, and proportions of tongue layers according to total tongue thickness.

Parameter	Total tongue thickness (µm)	Epithelial layer		Horny layer		Lamina propria/ submucosal layer		Stromal tissue percentage of Weber's salivary glands (%)
Group		Thickness (µm)	Proportion (%)	Thickness (µm)	Proportion (%)	Thickness (µm)	Proportion (%)	
Adult group	916.27± 0.64	135.42 ± 6.49	14.78 ± 0.71	70.62 ± 6.56	7.71 ± 0.72	40.81 ± 5.76	4.45 ± 0.63	7.82 ± 0.49
Aged group	740.76± 1.65*	83.02 ± 7.26*	11.21 ± 0.98*	26.72 ± 5.01*	3.61 ± 0.67*	116.89 ± 10.60*	15.78 ± 1.44*	19.15 ± 0.65*

Values are mean±SD. *p<0.05 vs adult group, as determined by Mann-Whitney U-test.

Table 2. Morphometric parameters of the tongue muscle layer of the study groups.

Parameter Group	Diameter of myofibers (µm)	Length of myonuclei (µm)	Length of sarcomeres (µm)	Number of sarcomeres/ mm
Adult group	35.47 ± 0.86	10.90 ± 0.61	2.03 ± 0.05	492.94 ± 12.62
Aged group	20.12 ± 1.53*	9.92 ± 0.54*	2.43 ± 0.02*	411.85 ± 3.90*

Values are mean±SD. *p<0.05 vs adult group, as determined by Mann-Whitney U-test.

diameter of lingual muscle fibers based on his histopathological evaluation.

Although the actual causes of aging are still unknown, theories were have been produced to explain these changes comprising the damage concept, including the accumulating damage due to DNA breaks, oxidized bases and/or mitochondrial malfunctions; or the programmed aging concept, including internal processes such as DNA telomere shortening (Youssef, 2017).

Reactive oxygen species (ROS) are the key mediators causing aging. According to this theory, oxidative damage occurs due to the over-production of ROS or due to lack of counteraction by the antioxidants (Simioni et al., 2018). With aging, ROS induce DNA damage and downstream damage (Robert and Wagner, 2020). So, it is believed that ROS could produce as many as 50,000 DNA lesions per cell per day contributing to aging (Maynard et al., 2015; Blagosklonny, 2008). In addition, mitochondrial dysfunction may be an important part of such aging process that is due to increasing ROS accumulation producing DNA damage of these organelles (Robert and Wagner, 2020). With the progression of these mitochondrial damaging events, these destructed mitochondria lead to increased ROS amount and produce less ATP during aging (Stefanatos and Sanz, 2018). Importantly, up-regulation of the antioxidative enzyme signaling pathways also has a critical role in decreasing the oxidative stress activity; however, at animal aging, it was proven that there is progressively diminished antioxidant system capacity encouraging the sequelae of ROS accumulation (Vina et al., 2006; Wei and Lee, 2002).

The progressive telomere shortening was the first developed molecular explanation for cell senescence that occurs progressively every cell division (Yousefzadeh et al., 2021). So, telomere length was considered to be the most suitable aging biomarkers during past decades because of its known role in cellular aging (Vaiserman and Krasnienkov, 2021). Despite DNA polymerase is the main responsible for DNA synthesis in the cell, it cannot efficiently replicate the last point of linear DNA leading to progressive telomere shortening every cell cycle (Jiang et al., 2007; Chim et al., 2018). At normal circumstances, telomere capping is essential for differentiation of the chromosomal ends from DNA breaks within the genome (Jiang et al., 2007). Also, oxidative stress is well-known to be implicated in aggravation of telomere shortening, and since telomeres prevent fusion of chromosome ends and also hinder the recognition of chromosomal ends as sites of DNA damage, ROS accumulation is a triggering factor for cellular senescence (Ledda et al., 2020; Jafri et al., 2016).

Aging of the oral mucosa is characterized by decreased epithelial thickness, decreased cellular density and reduced mitotic activity leading to decreased tissue regeneration and healing capacities (Abu Eid et al., 2012). In our study, we found that the lingual mucosa undergoes massive atrophy of its epithelial layer and its papillae, in addition to vascular congestion of the underlying connective tissue with aging. Furthermore, with histomorphometric analysis, there were decreased total epithelial and horny layer thicknesses, while the thickness of the subepithelial connective tissue was significantly increased with age.

Sarcopenia is defined as reduced skeletal muscle mass and strength. It prominently occurs during aging leading to dysphagia in the elderly (Kletzien

et al., 2013). This age-related tongue musculature degeneration is caused by multiple pathways leading to altered muscle fiber chemistry, reduced muscle fiber contraction ability, increased muscle fatigability, and decreased neuromuscular junction size and number (Kletzien et al., 2018). The intrinsic lingual muscles, in this study, showed various structural changes in form of a decreased diameter of their myofibers with decreased number of the sarcomeres and length of the myonuclei, in addition to intramuscular adipose cell accumulation that may refer to the functional disturbances that may affect the elderly concerning defective swallowing. In accordance with our results, Cullins and Connor (2017) reported that with aging the transverse and verticalis muscles in the old group had significantly smaller muscle fibers in 32 months old Fischer 344/Brown Norway rats.

Xerostomia is a common complaint of elderly people that provides a negative impact on their health (Nagler, 2004). Salivary hypofunction can ultimately lead to halitosis, dental caries, gingivitis, dry mouth, dry lips, dysgeusia, dysphagia, mastication problems, mucositis, oropharyngeal candidiasis, poorly fitting prostheses, sleeping difficulty, speech difficulty and traumatic oral lesions (Turner and Ship, 2007). Elsaied (2019) found that there are changes in the Weber's salivary glands included salivary duct lumen widening, stagnation of salivary secretion and slight vascular engorgement in aged rats. In the current study, we found that there is massive atrophy of the acinar cells and increasing fibrous stroma amount.

Conclusion: The present study emphasizes that aging in mice is associated with a wide range of changes affecting the mucosa and musculature of the tongue, in addition to affecting the Weber's salivary glands and lingual lymphoid tissue.

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