

AGE-RELATED CHANGES IN RAT TONGUE

Nikola Pirovski*, Dimitrinka Atanasova^{*,**}, Donka Keskinova^{***},
Nikolay Dimitrov*

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Abstract

The effects of ageing on the oral mucosa are believed to be relatively small. The purpose of this study was to identify morphological changes observed in rat tongue during ageing in 20-day-old, 3-month-old, 12-month-old, 28-month-old, and 48-month-old rats. Age-related changes in the thickness of the epithelium and lamina propria, the density of the elastic fibres and the number of mast cells were histomorphometrically analyzed. In 20-day-old rats, the stratified squamous epithelium and lamina propria thicknesses were thinnest, whereas with age, epithelial thickness reached its greatest thickness in 48-month-old rats and lamina propria thickness was most noticeable in 12-month-old rats. The arrangement and distribution of elastic fibres were disrupted in the lamina propria with ageing. The density of elastic fibres in lamina propria was lowest in 20-day-old rats, while in 12-month-old rats they reached their highest density, and then decreased sharply again in older rats. The number of mast cells per unit area was lowest in 20-day-old rats, reaching its highest number in 12-month-old rats and remaining high in older animals. In conclusion, with age, there was a moderate positive correlation and a statistically significant increase in tongue epithelial thickness, as well as a significant thickening of the lamina propria and an increase in the number of mast cells. The density of the elastic fibres in lamina propria decreased, but no negative correlation was observed with ageing.

Key words: rat tongue, ageing, epithelial thickness, mast cells

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Introduction. Ageing affects all systems and organs in the animal and human body. Rats are one of the most commonly used animals in experimental studies, in most cases using sexually mature 3-month-old rats. Due to the high mortality in adult rats, few animals live to 48 months of age. At this age, animals are difficult to move, some have partial loss of limb movement and many diseases. For this reason, research on such elderly animals is rare worldwide. There are no data on morphological examination of the tongue in 48-month-old rats, these are elderly rats that are thought to correspond to the human age of 120-year-olds. The oldest rats studied were 24 months [1] and 24–26 months [2]. In our previous studies, we obtained results for 12-month-old rats [3].

The dorsal surface of the tongue is partially covered by keratinized stratified squamous epithelium. The lamina propria is represented by the loose connective tissue with blood vessels and nerves. The lamina propria is fused with the underlying connective tissue of the tongue muscles without a sharp boundary. The papillae, which are of different size, shape and location, are located on the dorsal surface of the tongue [4]. The elastic fibre system constructing the lamina propria of the tongue in rats is composed of elastin and elastic fibres, whereas it lacked oxytalan fibres [5]. The striated muscle is disposed mainly in three planes arranged at right angles to one another. The blood vessels include arteries, arterioles, veins, venules, and capillaries. The nerves usually appear as small groups of a combination of myelinated and unmyelinated processes surrounded by a perineurium [6].

At present, there is convincing evidence that the keratinocytes of ageing rats showed a wide nuclear space in comparison with the cytoplasm. In addition, elongated mitochondria are observed at the periphery of the cells and near the nuclear space. Ultrastructural modifications in the epithelial cells of ageing animals have also been reported [1]. Lipid pigment (lipofuscin) was examined in the perioral and lingual muscles of ageing rats and a significant amount of lipofuscin was found in the muscles of the tongue in 24–26 month old rats [2].

An abundance of mast cells was found in the tongue of healthy rats and they accumulated around nerve fibres and blood vessels [3,6]. Mast cell (MC) function is mainly related to immunity, protection against parasitic infections, immunomodulation, tissue repair, and angiogenesis [7].

Rat mast cells were detected on the 15th day of fetal life. In the first days after birth, the number of mast cells remains unchanged. In the second week, a progressive and marked increase was observed, reaching significant values [8]. The distribution of mast cells in rat tongue has been studied by many authors, with differences in the presence and extent of age-related changes [3,8–10].

Age-related changes in the tongue of mice [11] and humans have been reported in the literature [12–15].

The aim of this study was to investigate the morphological changes observed in the tongue of rats with ageing. For the first time, we describe morphological

changes observed in the tongue of 48-month-old rats. Research on elderly rats is a challenge, as rats rarely live to that age and there is a high mortality rate. We believe that our research will provide new and valuable information on the morphological changes in rat tongue during ageing.

Materials and methods. Wistar rats of both sexes were used for experimental animals in the present study. The tongues of 20-day-old ($n = 5$), 3 month-old ($n = 5$), 12-month-old ($n = 5$), 28-month-old ($n = 5$) and 48-month-old rats ($n = 3$) were used. The rats were provided by project 13/2017 at the Medical Faculty of the Trakia University. The experiments were conducted in accordance with the regulations for work with experimental animals in the Republic of Bulgaria following a standard protocol established by the Bioethical Commission at the Department of Anatomy at the Medical Faculty of the Trakia University.

The animals were deeply anesthetized by intraperitoneal injection of xylazine and ketamine (40–80 mg/kg) and transcardially perfused first with 0.05 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. After perfusion, the tongue was cut near the root and a piece of it was placed for fixation in 10% formalin for 48 h. The samples were then dehydrated, embedded in paraffin and cut into 5 μm thick sections. Classical histological staining such as Hematoxylin and Eosin (H&E), Orcein staining for visualization of elastic fibres, Van Gieson for collagen fibres and Bismarck brown staining for mast cells were applied.

The samples were photographed with a research microscope (Leica DM1000) equipped with a digital camera (Leica DFC 290). The thickness of the epithelium and lamina propria were calculated from the dorsal part of the tongue. The number of mast cells was examined for an area of one square millimeter.

Statistical calculations were carried out using IBM SPSS Statistics (v.25) and significance was fixed at $p < 0.05$. All indicators were presented as mean and standard deviation (Mean $\pm$ SD). The verification of the normal distribution of observations by groups was performed with the Shapiro–Wilk Test. The verification of the influence of age on each of the studied indicators was performed with one-way analysis of variance (ANOVA). Depending on the presence of equality of variances in the age-comparable groups, a Bonferroni test for equal variances or a Games–Howell test for unequal variances was used for multiple pairwise comparisons. Spearman’s coefficient was used to measure the relationship between age and indicators.

Results. In our studies, we observed histological changes in the morphology of the tongue in rats with ageing. The changes were mainly in the epithelium, lamina propria, localization and distribution of collagen and elastic fibres in lamina propria and number of mast cells (Fig. 1, 2).

The results of the analysis show a statistically significant difference between the mean values at different ages for each of the four indicators (Table 1). The age of the rats located in the columns was identified with an alphabetical key.

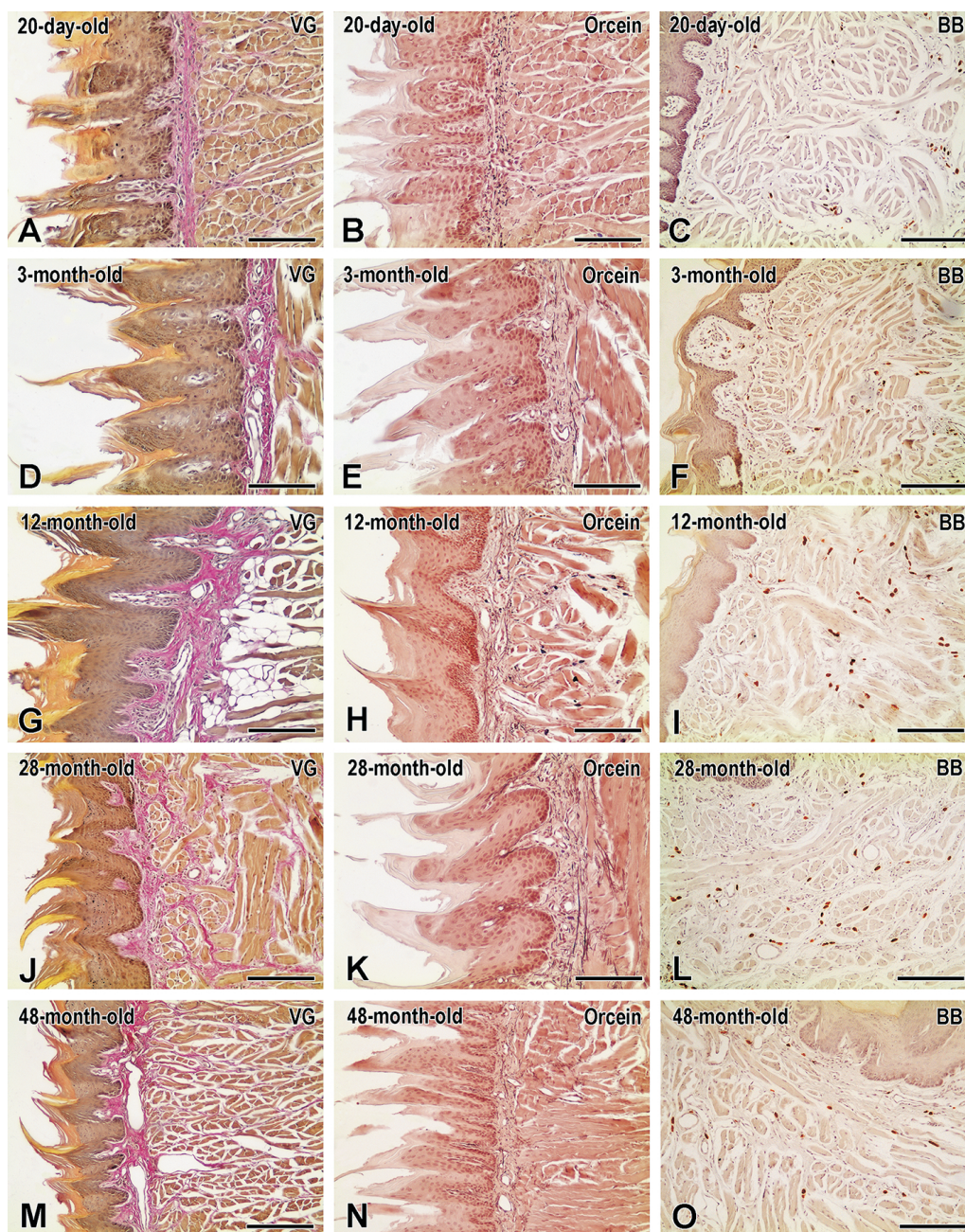


Fig. 1. Photomicrographs of the dorsal tongue in rats stained with Van Gieson (VG, left column), Orcein (central column) and Bismarck Brown (right column) in 20-day-old (A-C), 3-month-old (D-F), 12-month-old (G-I), 28-month-old (J-L) and 48-month-old rats (M-O). Scale bars: 50 μm

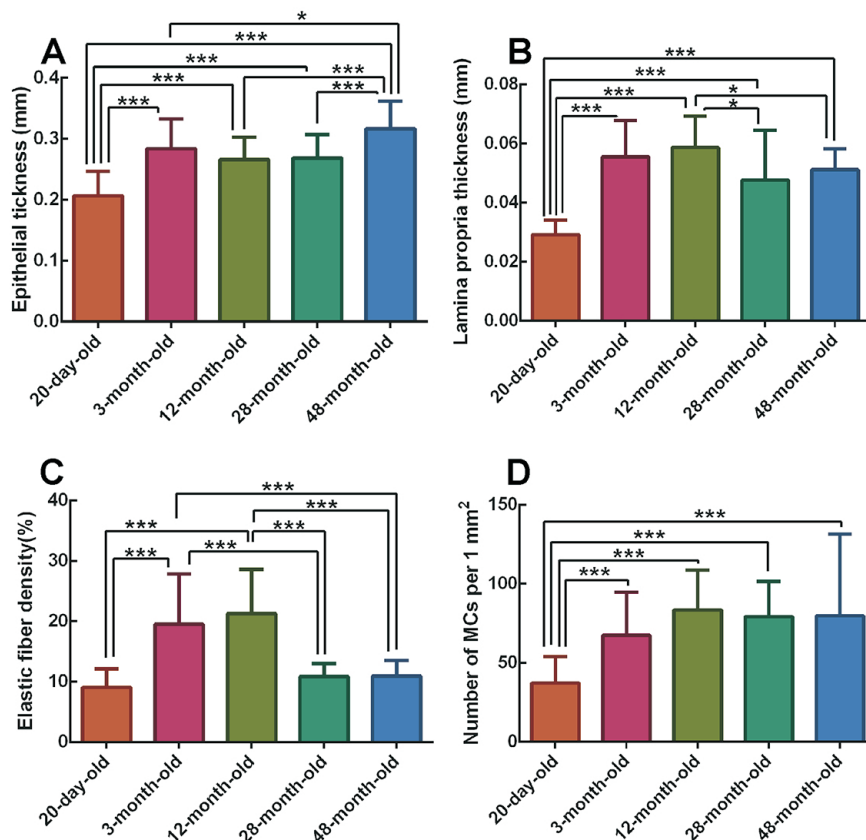


Fig. 2. Statistical comparison of epithelial (A) and lamina propria (B) thickness, elastic fibre density (C) and mast cell count (D) in five groups of experimental rats. The data represent mean values + SD. Asterisk indicates significant differences between age groups – * $p < 0.05$; *** $p < 0.001$

For each multiple pairwise comparison with a statistically significant difference ($p < 0.05$), below the average were placed the keys of the ages of which, this average was greater (Table 1).

In 20-day-old rats, stratified squamous epithelium and lamina propria thicknesses were thinnest (Fig. 1A, 2A), whereas with age, epithelial thickness reached its greatest thickness in 48-month-old rats (Fig. 1M; 2A; Table 1) and lamina propria thickness was most noticeable in 12-month-old rats (Fig. 1G; 2B; Table 1). The papillae were not as well formed in the elderly as in the sexually mature 3-month-old rats (Fig. 1D). Collagen fibres were more abundant in the muscle tissue of adult rats, and changes in the arrangement and organization of collagen fibres were observed in lamina propria (Fig. 1A, D, G, J, M). The arrangement and distribution of elastic and collagen fibres were disrupted in the lamina propria with ageing. The density of elastic fibres in lamina propria was lowest in

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An alphabetical key identifies the age of the rats from which the given value is greater. The given values represent the Mean and the Standard Deviation (Mean $\pm$ SD)

	20-day-old rats A	3-month-old rats B	12-month-old rats C	28-month-old rats D	48-month-old rats E
Epithelial thickness (mm)	0.207 $\pm$ 0.040	0.284 $\pm$ 0.048 A	0.266 $\pm$ 0.036 A	0.269 $\pm$ 0.038 A	0.317 $\pm$ 0.046 A B C D
Lamina propria thickness (mm)	0.029 $\pm$ 0.005	0.056 $\pm$ 0.012 A	0.059 $\pm$ 0.011 A D E	0.048 $\pm$ 0.017 A	0.051 $\pm$ 0.007 A
Elastic fibre density (%)	9.019 $\pm$ 3.024	19.541 $\pm$ 8.258 A D E	21.380 $\pm$ 7.176 A D E	10.865 $\pm$ 2.159	10.978 $\pm$ 2.485
Number of mast cells	37.356 $\pm$ 16.789	67.407 $\pm$ 27.252 A	83.584 $\pm$ 25.227 A	79.262 $\pm$ 22.101 A	79.713 $\pm$ 51.771 A

20-day-old rats (Fig. 1B; 2C; Table 1), while in 12-month-old rats they reached their highest density (Fig. 1H; 2C; Table 1) and then decreased again with age. The number of mast cells per unit area of 1 mm² was the lowest in 20-day-old rats (Fig. 1C; 2D; Table 1), reaching the highest number in 12-month-old rats, while with age their numbers remained high.

Spearman's Rho correlation analysis revealed the strong statistically significant positive correlation between epithelial thickness and age ($\rho = 0.522$, $p < 0.001$) (Fig. 3A). In addition to the observed relationship between epithelial thickness and age, we found a moderate positive correlation and pattern that with age, the thickness of the lamina propria increases ($\rho = 0.345$, $p < 0.001$) (Fig. 3B), as well as the number of mast cells ($\rho = 0.387$, $p < 0.001$) (Fig. 3D). With age, the density of elastic fibres decreases, but there was no clearly observed negative correlation between these two factors ($\rho = -0.013$, $p = 0.871$) (Fig. 3C).

Discussion. In our studies, we found that tongue epithelial thickness developed significantly in 3-month-old rats compared with 20-day-old rats, while in 12-month-old and 28-month-old animals, some thinning of the stratified squamous epithelium was observed. Surprisingly, in the oldest rats, we again found epithelial thickening, which was statistically significant compared to all other groups we studied, but it was quite possible that this deviation was due to the small sample of experimental animals available in elderly rats. The thickness of the lamina propria in 3-month-old rats was very well developed, reaching maximum development in 12-month-old animals. With ageing, some thinning of the lamina propria was observed in rats, but in general, there was a moderate positive correlation between the thickness of the lamina propria and age. The density and organization of elastic fibres in lamina propria was highest in 12-month-old rats, but with age in older rats, the density of elastic fibres decreased sharply and reached levels as in 20-day-old rats.

In the human tongue of the apex region in the elderly people morphological

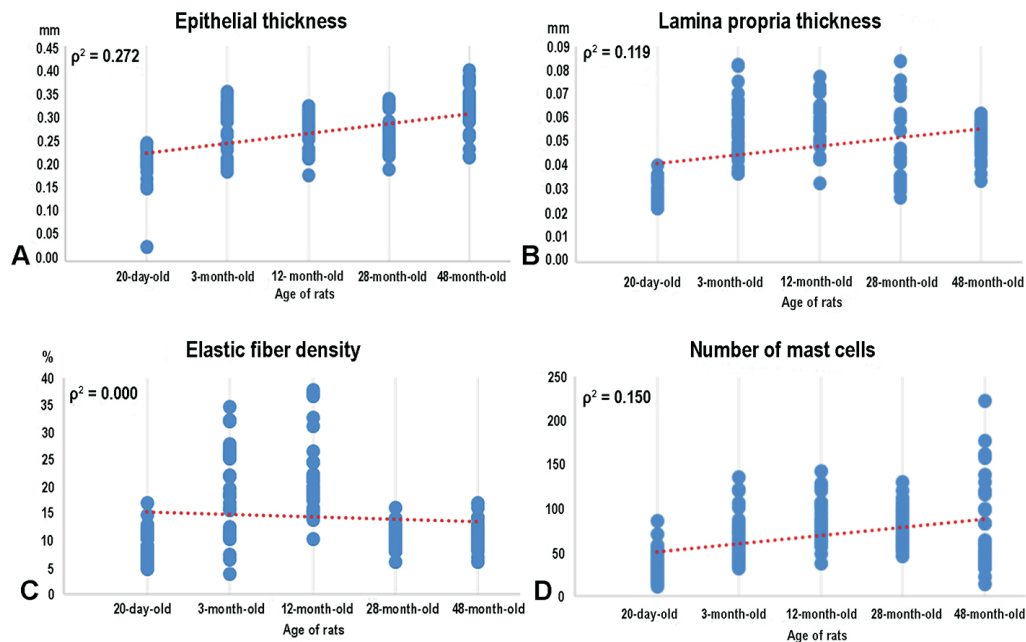


Fig. 3. Correlation between the Epithelial thickness (mm) (A), Lamina propria thickness (mm) (B), Elastic fibre density (%) (C) and Number of mast cells per 1 mm²(D) and age. Nonparametric Spearman rank coefficient (ρ) was used

changes of layers, fibrosis, structural changes of myocytes and myofibrils, nerves and neurofibrils were observed [15]. Lingual epithelium in the older humans is significantly thinner than that in the younger ones. The mean epithelial thickness decreased significantly with ageing [13]. In the human tongue the number, thickness and length of each secondary protrusion of connective tissue core of the filiform papilla decreases roughly proportionally with age. The connective tissue core of fungiform papillae, which have a corolliform structure, become thin with age [14].

In our studies, there is no clearly noticeable change in the papillae and connective tissue in the tongue. There was a relatively slight decrease in the volume and height of connective tissue in the papillae in adult rats.

Gradual reduction in epithelial thickness is a characteristic feature of mammalian ageing. An increase in mean epithelial thickness was observed in adult mice, followed by a gradual decrease to ageing. A noticeable increase in the keratin layer is observed with ageing as an adaptive response to a decrease in epithelial thickness [11]. In contrast to a study in adult mice, where an increase in the keratin layer of the epithelium was observed with age, in our studies in older rats we did not notice such a thing.

Previous research by some authors has suggested that there was no clear evidence of a difference between numbers of mast cells in young and older animals [9]. Many authors observe increased number of mast cells in different locations in the body in old age, due to the chronic inflammation process accompanying ageing [16]. In studies by other authors, it is claimed that the distribution of MCs in rat tongue was different in rats of different ages [3,8,10].

Mast cells play a crucial role in the ageing process of the skin in rats. In adult rats, a significant increase in skin levels of histamine and heparin was observed compared to control rats. In addition, there was a marked increase in intact and degranulated dermal mast cells compared to controls. The dermal collagen bundles are obviously reduced and appear distorted over a long distance [10].

Morphological changes in adult rats in the tongue are not as clear as described in elderly people. Epithelial and muscular atrophy were less pronounced in adult rats than in elderly people. Changes in elastic fibres and mast cell counts were more pronounced as differences in adult rats. This is probably due to the shorter lifespan of rats compared to humans and species specificity, or to the difference in the methodology used. This suggests that despite some agreement with some results, the results obtained in adult rats cannot be directly related to the age-related changes that occur in humans. Tongue ageing might not follow the same pattern as the rest of the organs. However, it is possible that age-related changes in tongue are associated with some of the tongue diseases that occur in adults.

The current study provides new data about the dynamics of thickness of tongue epithelium and lamina propria in the process of ageing based on morphometry and correlative analysis. With ageing, there is a thickening of the tongue epithelium well demonstrated by strong positive correlation. The thickening of lamina propria in adult rats is followed by some thinning during ageing in tandem with the decreased density of elastic fibres in the lamina propria. However, a moderate positive correlation was established between age and thickness of the lamina propria. The increase in the number of mast cells during ageing suggests the vulnerability of the tongue to immune and inflammatory processes.

Conclusion. In conclusion, with ageing, there is a thickening of the tongue epithelium and lamina propria, as well as an increase in the number of mast cells.

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**Department of Anatomy*
Faculty of Medicine
Trakia University
11, Armeyska St
6003 Stara Zagora, Bulgaria
e-mail: nikola.pirovski@trakia-uni.bg
nikolay.dimitrov@trakia-uni.bg
dimitrinka.atanasova-dimitrova@trakia-uni.bg

***Institute of Neurobiology*
Bulgarian Academy of Sciences
Akad. G. Bonchev St, Bl. 23
1113 Sofia, Bulgaria
e-mail: d.atanasova@inb.bas.bg

****Department of Applied and Institutional Sociology*
Faculty of Philosophy and History
University of Plovdiv "Paisii Hilendarski"
4000 Plovdiv, Bulgaria
e-mail: d.keskinova@uni-plovdiv.bg