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Short Communication

## Effects of Aging on Mouse Tongue Epithelium Focusing on Cell Proliferation Rate and Morphological Aspects

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### Abstract

The aim of this study was to investigate cell proliferation rate and certain morphological features of mouse epithelium as aging progresses. Tongue biopsies were performed on female mice (*Mus domesticus domesticus*) at 2, 8, 14 and 20 months of age as indicative of adolescence, adulthood, early senescence and senescence, respectively. Histological sections of tongue were stained with hematoxylin-eosin and subjected to silver staining for active nucleolar organizer region counting. Cell proliferation rate and epithelial thickness analysis were carried out. Analysis of variance detected no differences between the groups in terms of numbers of silver-stained dots associated with nucleolar proteins. There was an increase in mean epithelial thickness in adult animals, followed by a gradual reduction until senescence. Mean keratin thickness presented an increase at 8 and 20 months of age. This difference is probably related to puberty, growth or dietary habits. Aging has no influence on oral epithelial proliferation rate in mice. A gradual reduction in epithelial thickness is a feature of aging in mammals. A conspicuous increase in the keratin layer was observed in senescence as an adaptative response to the reduction in epithelial thickness. These results suggest that aging affects the oral epithelium maturation process through a mechanism that is not related to cell proliferation.

Key words: Aging—Morphology—Proliferative activity—Silver staining—Tongue mucosa

### Introduction

Aging affects many tissues and organs throughout the body<sup>7,16,25</sup>, but there are currently disagreements about certain clinical and histological data concerning its effects on the oral mucosa<sup>6,13,15,19,24</sup>.

The major function of the oral mucosa is to protect the deeper tissues and organs in the

oral cavity. Oral epithelium plays a role as a permeability barrier lining the oral cavity<sup>22</sup>.

Some studies have analyzed the effect of aging on epithelial renewal. Nevertheless, there is no consensus in the literature on how epithelial maturation and proliferation are affected by age<sup>6,13,15,19,24</sup>. Çelenligil-Nazliel *et al.*<sup>4</sup> did not find differences in epithelial renewal. However, aging is related to a

gradual reduction in connective tissue turnover. According to Cleaton-Jones<sup>6)</sup>, oral epithelial proliferation is reduced in the elderly. On the other hand, another study states that this activity increases with age<sup>24)</sup>. Zimmermann and Zimmermann<sup>26)</sup> found a reduction in the epithelium keratinization of the hard palate and the gingiva in older individuals. Nevertheless, there are no studies in the literature that have analyzed a single group of animals over time.

Nucleolar Organizer Regions (NORs) are loops of DNA that, in humans, are located on the short arm of acrocentric chromosomes 13, 14, 15, 21 and 22, and codify ribosomal RNA<sup>12)</sup>. AgNORs are acidic proteins that have an affinity for silver and are associated with active NORs in the interphase. AgNORs appear as black dots inside the yellow-brownish nucleus, and their number is related to cell proliferation rate<sup>10)</sup>.

The aim of this study was to analyze the effects of aging on cell proliferation rate and some aspects of the morphology of mouse tongue epithelium.

## Materials and Methods

This study was a randomized blind trial. The study sample comprised twenty CF1 female mice (*Mus domesticus domesticus*). Animals were fed a standard laboratory chow diet for mice (Nuvilab/CRI, Nuvital Nutrientes LTDA, Colombo, Brazil) and water *ad libitum*.

Tissue samples were collected at 2, 8, 14 and 20 months as representative of adolescence, adulthood, early senescence and senescence, respectively. For this procedure, the mice were anesthetized in an anesthetic chamber by inhalation of diethyl ether (Ethyl Ether Techn—imported by Importadora Química Delaware Ltda., Porto Alegre, Brazil). A 3-mm punch biopsy was then taken from the center of the tongue dorsum<sup>3)</sup>.

All specimens were fixed in 10% neutral buffered formalin for 24 hrs and embedded in paraffin in transverse orientation. Two 4- $\mu$ m histological sections were obtained from each

paraffin block (RM2125 microtome, Leica Microsystems<sup>®</sup>, Solms, Germany); one was hematoxylin-eosin (HE) stained, and the other was submitted to silver-staining for AgNOR counting according to the protocol described by Ploton *et al.*<sup>14)</sup>.

### 1. Cell proliferation rate—AgNOR technique

Images were captured using a video camera (TK-C620, 1 CCD, Victor Co., Tokyo, Japan) coupled to a binocular microscope (model CH30RF100, Olympus Optical Co., Ltd., Tokyo, Japan) with 1,000 $\times$  magnification. The images were captured so that both basal and suprabasal cells could be seen, starting from the border section. Images were recorded for 30 consecutive microscopic fields or for all microscopic fields when there were fewer than 30. Basal cells were characterized by the presence of at least one contact point with the adjacent basement membrane. Suprabasal layer cells are more superficial than basal layer cells. However, they do not present the flattened shape that is typical of cells in the superficial layer itself<sup>3,19)</sup>.

The number of AgNOR dots per nucleus in the tongue epithelium was quantified by visual count (Fig. 1). One hundred cells per slide were evaluated: 50 basal layer cells and 50 suprabasal layer cells<sup>1)</sup>. Cells with overlapping or folded nuclei and areas with artifacts were excluded from the analysis<sup>3)</sup>. Overlapping dots were counted as one dot according to the method established by Crocker *et al.*<sup>9)</sup>. Measuring procedures were adjusted before quantification, and the same adjustment procedures were repeated before the end of data collection (kappa coefficient;  $p > 0.7$ ).

### 2. Morphometric analysis—Hematoxylin/Eosin

Epithelial thickness was measured from the basal membrane to the granular layer and used to indicate proliferative activity in 3 microscopic fields per slide with 400 $\times$  magnification. Keratin layer thickness was measured at the tip of the filiform papillae and used as an indicator of epithelial maturation/keratinization (Fig. 2). The linear measurement

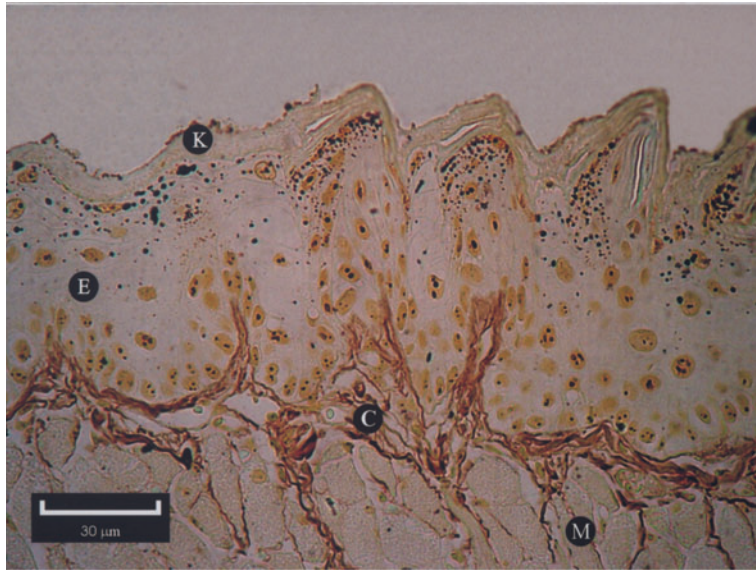


Fig. 1 Photomicrograph of tongue mucosa, silver-stained, showing keratin layer (K), epithelial tissue (E), connective tissue (C) and muscle tissue (M) (AgNOR technique,  $\times 400$ ).

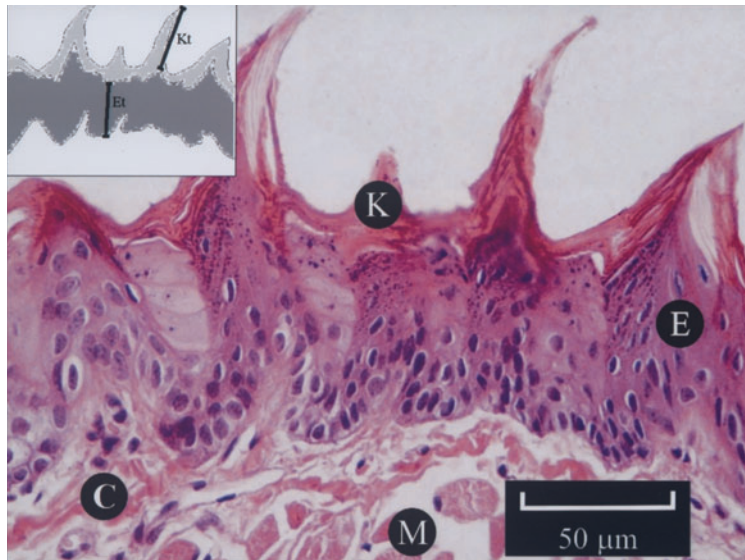


Fig. 2 Tongue mucosa, showing epithelial tissue (E), connective tissue (C) and muscle tissue (M). Epithelium thickness (Et) was measured from basal membrane to epithelial granular layer. Keratin layer thickness (Kt) was measured from end of granular layer to tip of filiform papillae (H-E,  $\times 400$ ).

tool available in the ImageTool 3.0 (University of Texas, San Antonio, USA) was used for the analysis, and the adjustment procedures were repeated before the end of data collec-

tion (Student's *t*-test;  $p > 0.7$ ).

### 3. Statistical analysis

Data were expressed as mean  $\pm$  SD. Analyses

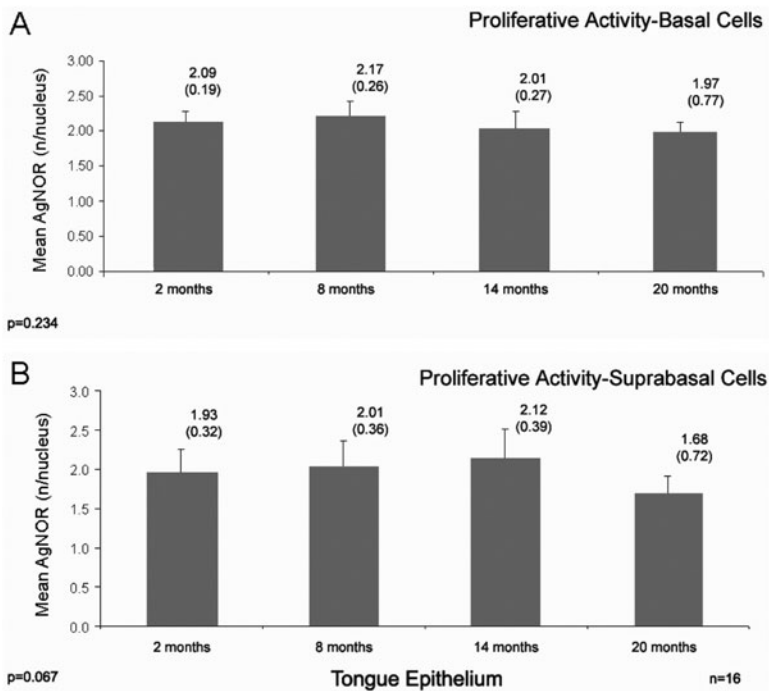


Fig. 3 Cell proliferation rate analysis by AgNOR count  
 (A) Evaluation of basal layer cells. (B) Evaluation of suprabasal layer cells.

were performed by an examiner unaware of which group each image belonged to. An analysis of variance (ANOVA) and least significant difference (LSD) for multiple comparison tests were used to compare the groups.

#### 4. Ethical considerations

This study was approved by the Ethics Committee at the School of Dentistry, Universidade Federal do Rio Grande do Sul under protocol number PPGO 40/99.

### Results

Evaluation of AgNOR counts did not reveal any significant differences in the cell proliferation rate of mouse tongue epithelium between different age groups, either for the basal ( $p=0.234$ ) or for the suprabasal layer cells ( $p=0.067$ ) (Fig. 3A and 3B).

Epithelial layer thickness (Fig. 4A) exhibited

an increase up to adulthood and then a gradual decrease from adulthood into senescence. This decrease was statistically significant only when adult animals were compared to senescent ones.

Keratin layer thickness exhibited a certain increase (Fig. 4B) up to adulthood and there were variations from adulthood until senescence, but these were not statistically significant.

### Discussion

Aging is a complex phenomenon. This study was designed to investigate the effects of aging on certain aspects of the morphology and cell proliferation rate of mouse tongue epithelium. A single population was followed up, making this a longitudinal study and minimizing the chances of individual characteristics influencing the results.

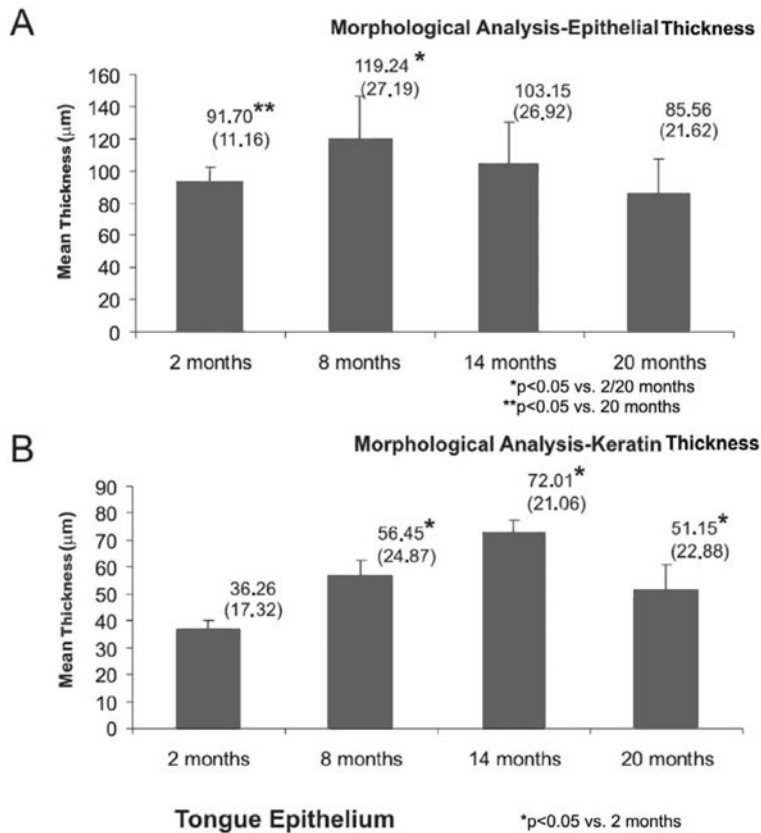


Fig. 4 Morphometric analysis

(A) Epithelial thickness measurements. Data represent mean  $\pm$  standard deviation ( $p < 0.05$ ; Analysis of Variance-ANOVA, Tukey's post hoc test; asterisk indicates statistically different groups). (B) Keratin thickness measurements. Data represent mean  $\pm$  standard deviation ( $p < 0.05$ ; Analysis of Variance-ANOVA, Tukey's post hoc test; asterisk indicates statistically different groups).

Many studies have evaluated two experimental times, one in adolescence and the other in senescence<sup>6,15,17,19,24</sup>. This could generate misunderstandings, as observed in this study. This study intended to take this bias into account by evaluating changes along different stages of life. By also studying groups at intermediate ages, it is possible to distinguish which changes are specifically related to aging. Toto *et al.*<sup>24</sup> used 15-month-old rats as their 'elderly group'. This period of time might not be long enough for the animals to be considered as old, since rats live for about 3 years. Our study took the precaution of using 20-month-old mice, which is a reason-

able age, since mice have a life span of about 20 to 25 months.

No significant difference could be detected between the study groups in terms of cell proliferation rate (Fig. 3). These findings confirm work by Scott *et al.*<sup>19</sup>, Çelenligil-Nazliel *et al.*<sup>4</sup> and Sakai *et al.*<sup>15</sup>. According to Çelenligil-Nazliel *et al.*<sup>4</sup>, the only difference caused by aging is a reduction in conjunctive tissue cell turnover due to increased apoptosis. On the other hand, some studies have found a reduction in cell proliferation<sup>6,24</sup>. However, those studies used a different technique to evaluate proliferation, making it difficult to compare results. The AgNOR technique has been

used for over 20 years and, as such, its relationship with cell proliferation rate is well established<sup>2,5,8,10–12,14,18,20,21,23</sup>. Çelenligil-Nazliel *et al.*<sup>4</sup>) and Sakai *et al.*<sup>15</sup>) used PCNA and BrdU, respectively, which are markers that are directly related to cell proliferation expressed during the interphase. The AgNOR technique has an advantage over other proliferation markers such as Ki-67, PCNA, tritiated thymidine and BrdU, since it provides information about cell proliferation rate, *i.e.*, cell doubling time, whereas the other proliferation markers mentioned indicate whether cells are dividing or not, *i.e.*, the growth fraction<sup>10,11</sup>). This advantage is the basis for choosing AgNOR as a more reliable marker of cell proliferation rate.

With regards to morphology, we observed an increase in the thickness of epithelium (Fig. 4A) and keratin (Fig. 4B) in adulthood, which was considered indicative of puberty and/or the growth process. Epithelial layer thickness (Fig. 4A) then exhibited a gradual decrease from adulthood into senescence. Based on this result, it may be speculated that this alteration makes epithelial tissue more susceptible to noxious agents. This decrease was conspicuous in the elderly (20-month-old animals), and the increase in keratin layer thickness in this group (Fig. 4B) may be a compensatory attempt to maintain tissue homeostasis. Hence, it is reasonable to suggest that aging affects the maturation process of oral epithelium, but that the cell proliferation rate increase should not be considered to be the source of this change. Sasaki<sup>17</sup>) examined human autopsy findings and found a similar relationship between reduced epithelial thickness and aging. Our findings corroborate his results: that aging is responsible for a morphological change in epithelium through a mechanism that is independent from changes in cell proliferation rate.

It was concluded that aging has no effect on the proliferation rate of mouse tongue epithelial cells. On the other hand, the maturation process of mouse tongue oral epithelium exhibits changes as aging progresses. It is reasonable to suggest that these changes to the maturation process could reduce the

capacity of the oral epithelium to act as a barrier, increasing the susceptibility of the oral mucosa to injuries.

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