Investigating cellular senescence in the aging tongue

Surbhi Chugh

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Jonathan An

Milda Chmieliauskaite

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Abstract

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Surbhi Chugh

Co-Chairs of the Supervisory Committee:

Jonathan An

Department of Oral Health Sciences

Milda Chmieliauskaite

Department of Oral Medicine

Background: Cellular senescence is a biological phenomenon that occurs at the molecular level in certain cells, wherein they stop to divide, persist, and damage nearby healthy cells by the production of a senescence-associated secretory phenotype (SASP). With age, various tissues in our body accumulate senescent or 'zombie' cells. On one hand, cellular senescence inhibits the growth of cancerous cells, and, on the other hand, SASP can cause tissue disruption and age-related diseases such as cancer. Within the oral cavity, the tongue has a high risk of decline and cancer with age. While multiple studies have explored the aging tongue in the context of cancer, there are limited studies evaluating the healthy, aging tongue.

Aim: The aim of this study was to evaluate whether the process of cellular senescence occurs within healthy, aged tongue tissues. Our hypothesis is that the healthy aging tongue will undergo cellular senescence.

Methods: Tongue specimens from young female primates (4-6 years, n = 3), old female primates (17–19 years old, n = 3), and old female primates treated with rapamycin (17–19 years old, n = 4) were obtained, and a tissue sample from the posterolateral area was taken. Western blot analysis was done to evaluate p16ink4a (p16) and p21WAF1/CIP1 (p21) expression levels, while qRT-PCR was completed to evaluate SASP markers. Histology slides were prepared for standard hematoxylin/eosin and lipofuscin staining. Statistical analysis was done using a one-way ANOVA test to compare the means of p16, p21, and SASP markers.

Results: Aged non-human primate (NHP) tongues showed a significant increase in the cell cycle arrest markers p21 and IL1A. Additionally, rapamycin reduced the elevated levels of IL1A e xpression. While qualitative tissue morphological changes demonstrated no indication of pathology, older NHP tongues had a thickened epithelial tissue layer and a greater number of rete ridges and lipofuscin granules compared to the younger group.

Conclusion: While literature demonstrates that cancerous tongues have an increase in cellular senescence markers, we show here that normal, aged tongues also have an increased cellular senescence, albeit without pathology. Further, the increase in IL1A was blunted by rapamycin treatment. Additional research is needed to understand the significance of IL1A attenuation in the

tongue for health and disease. Understanding cellular senescence may provide another way to evaluate the aging tongue beyond the clinical exam.

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I. INTRODUCTION

With a rapidly growing and aging population, there is a corresponding increase in the incidence of age-associated oral decline and disease, such as periodontitis, oral cancer, and xerostomia. [1] The tongue functions as a digestive organ and is an important accessory organ in chewing, swallowing, breathing, and speech. [4,5] The dorsum (upper surface) contains over 5000 taste buds and these cells, along with the epithelium undergo continual turnover, while the overall tongue is richly supplied with nerves and blood vessels. [6] During age, physiological changes, such as the decrease in thickness of the tongue epithelia or atrophy of the tongue acinar glands, lead to taste disturbances and poor mechanism of swallowing. [7] Oral cancer is also more prevalent in older adults, with the median age of diagnosis at 63 years [2] and is the most common site for oral cancer. [3] Despite age-related changes and a higher risk for tongue cancer, clinical evaluation primarily involves a visual inspection and palpation of its surfaces and care comes only after a disease is detected. However, studying the underlying biological process of aging in the tongue could provide additional perspectives on age-related diseases in older adults to guide future prevention and treatment efforts.

A major hallmark of aging is cellular senescence, a phenomenon which occurs at the molecular level in certain cells referred to as senescent cells, or "zombie cells". [8,20] Senescent cells are critical during both normal physiological states and pathological conditions. For example, senescent cells can prevent tumor growth or aid in tissue remodeling during wound healing. [8] On the other hand, with age, a variety of tissues in our body accumulate senescent cells, [36] which release a collection of molecules called senescence-associated secretory phenotypes (SASP). [24] SASP is a combination of pro-inflammatory cytokines, chemokines, and proteins that can cause damage to nearby tissues. [14]

Cells that express senescence markers accumulate with age in a variety of vertebrates, including mice, [63] non-human primates and humans, especially in highly renewable tissues such as the stroma and epithelia[9] Interestingly, studies have shown that elimination of these senescent cells in aged and diseased mice has been associated with improvements in health span and lifespan. For example, it has been demonstrated that $p16^{ink4a}$ (p16), a tumor suppressor gene, accumulates during adulthood and promotes age-dependent changes in several organs, such as the heart and kidney.[10] Clearing p16 cells genetically in aged mice has been shown to delay tumorigenesis and attenuate age-related deterioration of several organs, including the kidney, and fat. [8,49] Pharmacologically targeting senescent cells, via Dasatinib+Quercetin (D + Q), has been shown to reduce senescent cell abundance that decreases proinflammatory cytokine secretion in human adipose tissue and extends lifespan in aged mice.[11] In addition to genetically and pharmacologically clearing senescent cells, studies have also shown that targeting the mechanistic target of rapamycin (mTOR), [19] a major pathway during the aging process that regulates cell growth and metabolism, via rapamycin, may also impact cellular senescence.

The drug rapamycin specifically targets and inhibits mTOR signaling. It has shown to postpone numerous age-related pathological occurrences such as reducing the progression of different cancers such as skin, breast, pancreatic due to its inhibitory action on cell growth and proliferation in mice, as well as extending their lifespan. [12] Recent data have also demonstrated that blocking the mTOR pathway via rapamycin can decrease SASP. [10,13] In addition, rapamycin has been demonstrated to dampen the inflammatory characteristics of senescent cells by selectively inhibiting the translation of major SASP marker, IL1A.[13]

Overall, as the tongue is a highly renewable tissue, it is expected to undergo cellular senescence with age, but no studies have evaluated this phenomenon. Past studies evaluating

cellular senescence have been limited to tongues already expressing pathology as these are routinely biopsied. Taking biopsies of healthy, aged tongue in humans is not common. To overcome this challenge, non-human primate models (NHPs) provide a unique opportunity to study the healthy aging tongue. NHPs are an animal model that shares genetic similarity with humans, making them biologically more relevant than other animal models. [15-18] They also exhibit physiological similarities to humans in various organ systems, including the oral cavity. [15,16] Thus, evaluating the process of cellular senescence and targeting the senescence pathway in the aging tongue of NHPs may be more translationally relevant to aging humans.

II. AIMS & HYPOTHESES

Aim 1: Compare protein and immunohistochemical expression of p16, p21 in the tongues of young, old NHPs and old NHPs treated with rapamycin. We hypothesize that senescence markers will be upregulated in old NHP tongue compared to young NHP tongues.

Aim 2: Evaluate the expression of senescence-associated secretory phenotype (SASP) in the tongues of young, old NHPs and old NHPs treated with rapamycin. We hypothesize that SASP will be upregulated, and rapamycin will reduce levels of cellular senescence in old NHP tongues treated with rapamycin.

Aim 3: Compare tongue tissue samples of aged, young and aged treated with rapamycin using standard histology. We hypothesize that the aged tongue will show thinning of the epithelium, decrease in the number of rete ridges, and an increase in lipofuscin granules, like what has been reported in aged humans.

III. METHODS

Tongues of NHPs were procured from the Oregon National Primate Research Center. n. Young female NHP (4-6 years old, ~12-18 year-old human, n=3), aged female NHP (17-19 years old, ~52-58 year old human, n=3) and aged female NHP treated with rapamycin (17-19 years old, ~52-58 year old human, n=4) injected with 0.2mg/kg twice daily IM for 10 months were all provided by Oregon Health & Sciences University through Dr. Jonathan An's collaboration with Dr. Mary Zelinski and Dr. Steven Kohama.

Western Blot and Quantification of Cellular Senescence:

Samples from the posterolateral tongues of NHPs were excised, and the epithelial and muscle layers were segregated and stored at -80 °C. Each tissue layer was cryopulverized, and protein was extracted using RIPA. Quantification of protein was completed using either BCAA (Thermo) or the Quibit4 Fluorometer. Western blot was completed using 10% Bis-Tris Gel (MIDI, Bio-Rad) and transferred using Turbo Transfer (Bio-Rad). Antibodies $p16^{ink4a}$ (p16), $p21^{WAF1/CIP1}$(p21) (Cell Signaling), and GAPDH (Cell Signaling) were quantified using iBright (Invitrogen).

Quantitative PCR:

RNA extraction was completed using the Monarch Total RNA Kit (New England Biolabs). Quantification and validation of RNA was completed using Nanodrop/QuBit 4. cDNA was fabricated using iSCRIPT cDNA synthesis (Bio-Rad), and primers (IL1A, IL1B, IL6, IL8, TNF) were customized directly from the Bio-Rad PrimePCR Bank.

Histology:

Tissues were processed and embedded using standard histological techniques. Serial sections of 5 μm thickness were collected in the sagittal plane. Sections were stained for standard Hematoxylin and Eosin (H&E), and Sudan Black B for presence of lipofuscins granules. Slides were examined on the AmScope Microscope and images were taken using the Leica K3. The thickness of the epithelial tissues, number of rete ridges and density of lipofuscin granules were qualitatively assessed.

DATA ANALYSIS:

Statistical analysis was done using one way ANOVA test to compare means of p16, p21, and SASP markers that were assessed and compared among all the three different groups which were independent of each other. P value was set at $p \leq 0.05$. Graphs of p16, p21 and the different SASP markers (IL1A, IL1B, IL6, IL8, TNF) were plotted separately for epithelial and muscle tissues relative to GAPDH and β-actin using GraphPad Prism 10.

IV. RESULTS

Western blot expression for the cell cycle checkpoints p16, p21 was evaluated separately for epithelial and muscle tissue layers (Fig. 1a). P16 levels were elevated only in the muscle tissue samples of aged and the aged samples treated with rapamycin, however none of these were statistically significant (Fig. 1b). p21 levels were found to be elevated in the aged tongue samples

of both the epithelial and muscle tissue layers, when compared with the young NHP samples (Fig. 1c). p21 levels were found to be statistically significant only in the epithelial tissue samples of both the aged and the aged samples treated with rapamycin relative to the group of young NHPs (Fig. 1c)

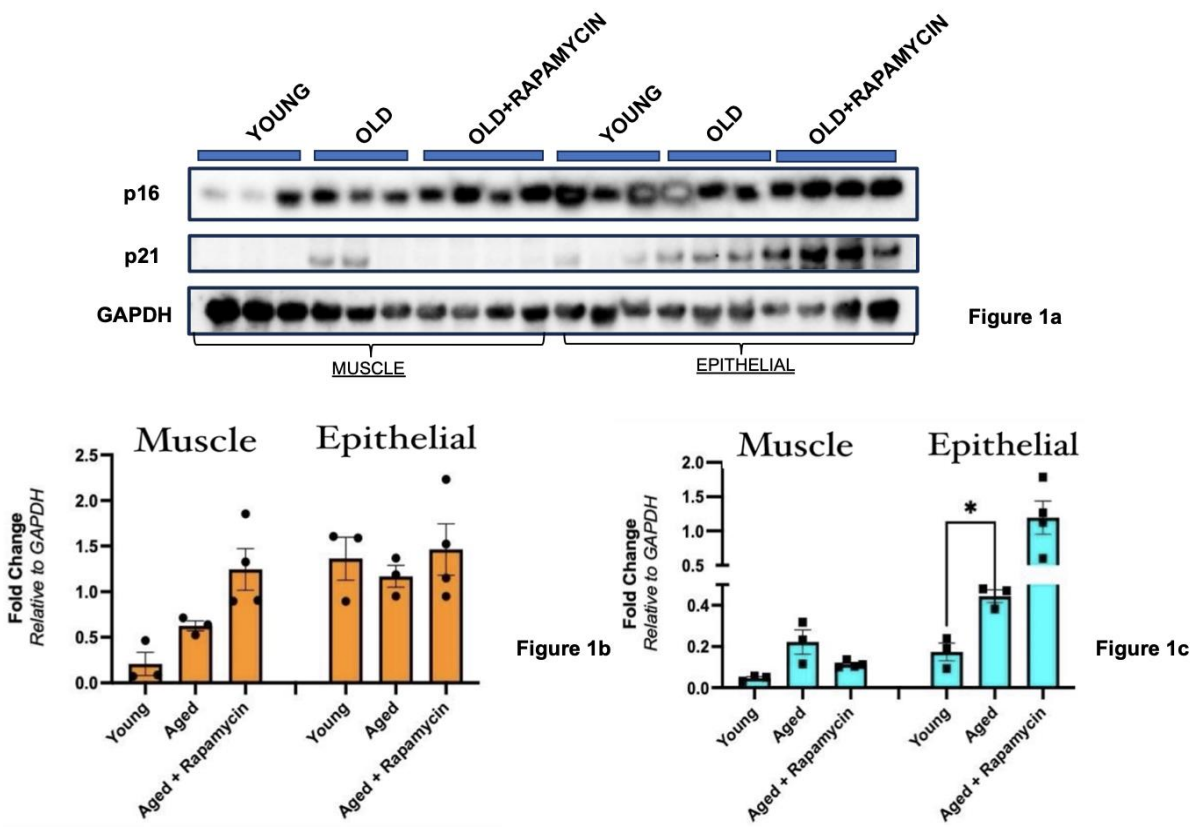

Figure 1a: Western blots show expression of p16, p21 and GAPDH (control) depicted for young, old and old treated with rapamycin groups. Figure 1b: Graph plotted using GraphPad for p16 expression relative to GAPDH showing muscle and epithelial tissue layers in young, aged and aged treated with rapamycin. Figure 1c: Graph plotted for p21 expression relative to GAPDH showing muscle and epithelial tissue layers in young, aged and aged treated with rapamycin. X axis represents the 3 study groups, and Y axis represents the fold change in both the plotted graphs, *P value: P<0.05.

The qRT-PCR results of the SASP markers of the epithelial and muscle tissue layers; while IL1A, IL1B, and IL6 were compared in the epithelial layer (Fig. 2a); IL1A, IL1B, IL8 and TNFα were the only markers showing a significant trend in the muscle layer (Fig. 2b) among the young, aged and aged tongue samples treated with rapamycin. IL1A showed a significant upregulation in the epithelial layer of the aged tongue compared to the young, while rapamycin treatment in the aged group significantly blunted these levels ($p<0.01$) (Fig. 2a).

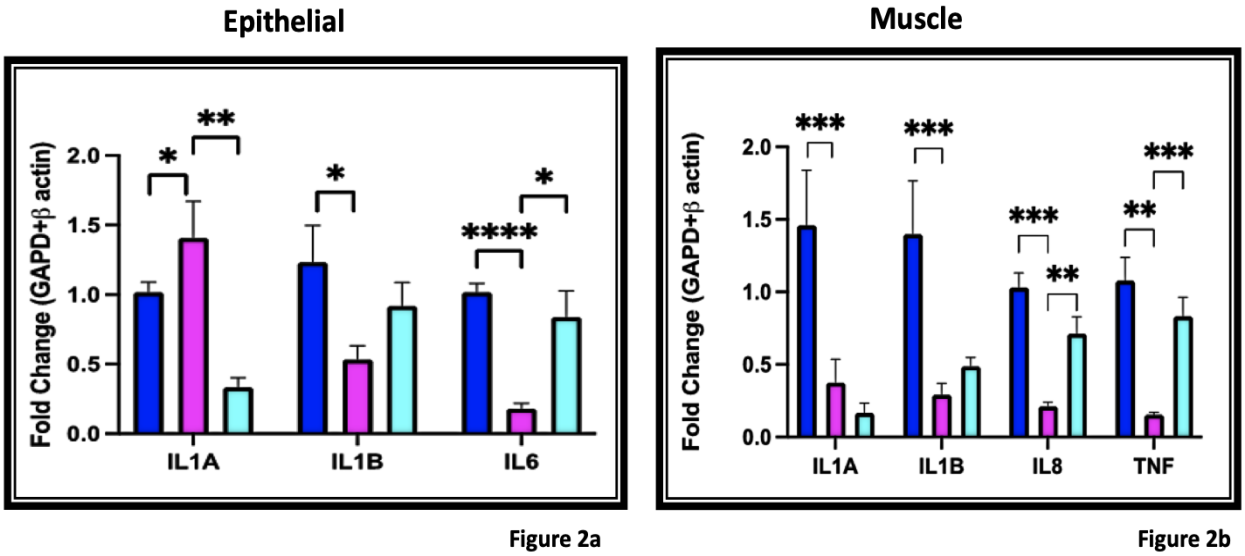

Figure 2a

Figure 2b

Figure 2a: Graphic representation of the specific SASP markers evaluated in the epithelial tissue where the X axis represents IL1A, IL1B and IL6 expression for the 3 study groups and Y axis represents the fold change relative to GAPDH and β-actin (control). Figure 2b: Specific SASP markers evaluated in the muscle tissue. X axis shows IL1A, IL1B and IL8 and TNF expression for the 3 study groups and Y axis shows the fold change relative to GAPDH and β-actin. Statistical analysis was completed using ANOVA, with p values <0.05 were considered statistically significant.*p<0.05, **p<0.01, ***p<0.001,****p<0.0001.

Young
Old
Old + Rapamycin (IM)

In addition, IL1B levels were observed to be downregulated in the old epithelial tongue samples with respect to the young group ($p<0.05$) and similarly IL6 levels were also shown to be downregulated in the group of old NHP's epithelial layer ($p<0.0001$) while IL6 levels in the rapamycin treated group were upregulated when compared to the old specimens ($p<0.05$). α all the SASP markers that were tested showed downregulation in the muscle layer of the aged tongue compared to the young, while rapamycin treatment in the aged group caused blunting of only IL1A levels when compared to its old counterparts (Fig. 2b).

Histological analysis of young NHP showed a well-defined stratified squamous epithelial tissue layer present, with distinct rete ridges (Fig. 3a). When compared to young NHP, the old NHP had a well-defined thicker epithelial tissue layer along with a greater number of distinct rete ridges (Fig 3b).

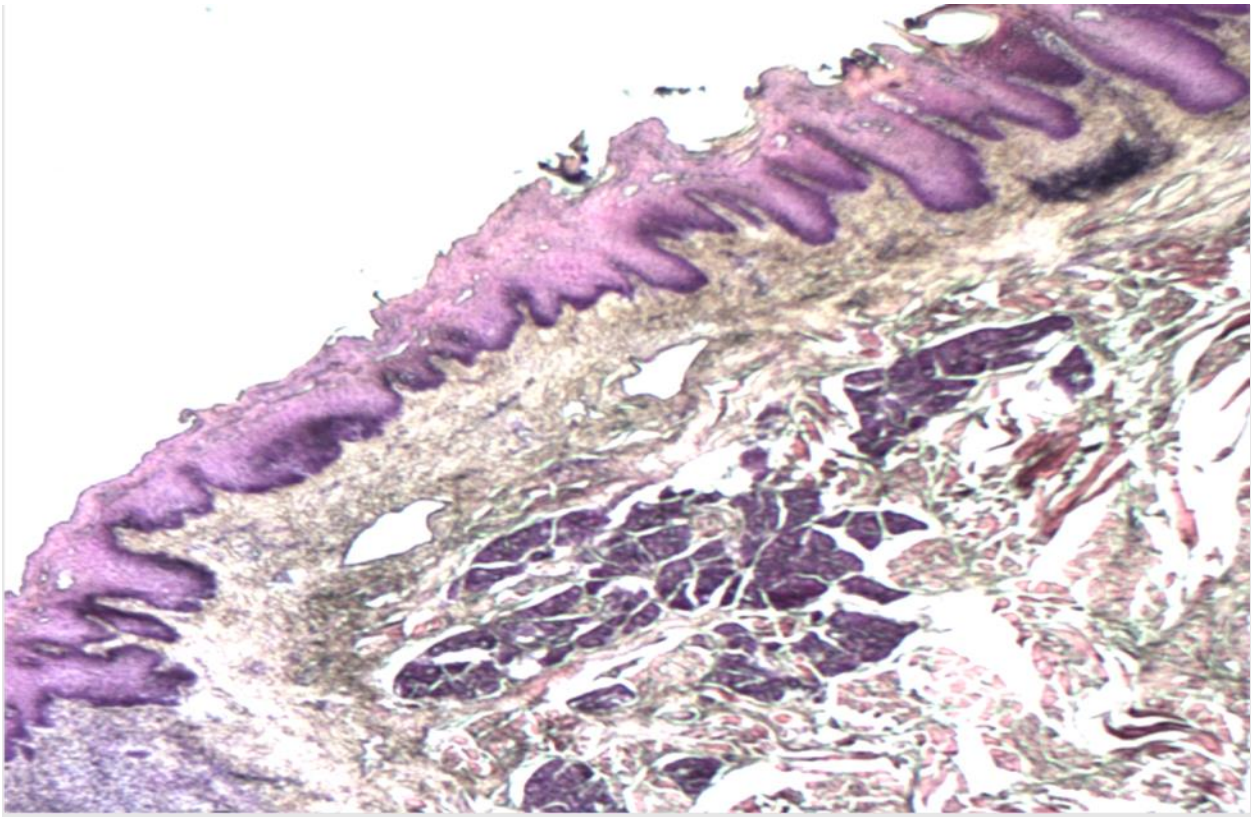

Figure 3a: **Histology slide of a young NHP (4X magnification)** showing epithelial tissue layer with rete ridges and connective tissue.

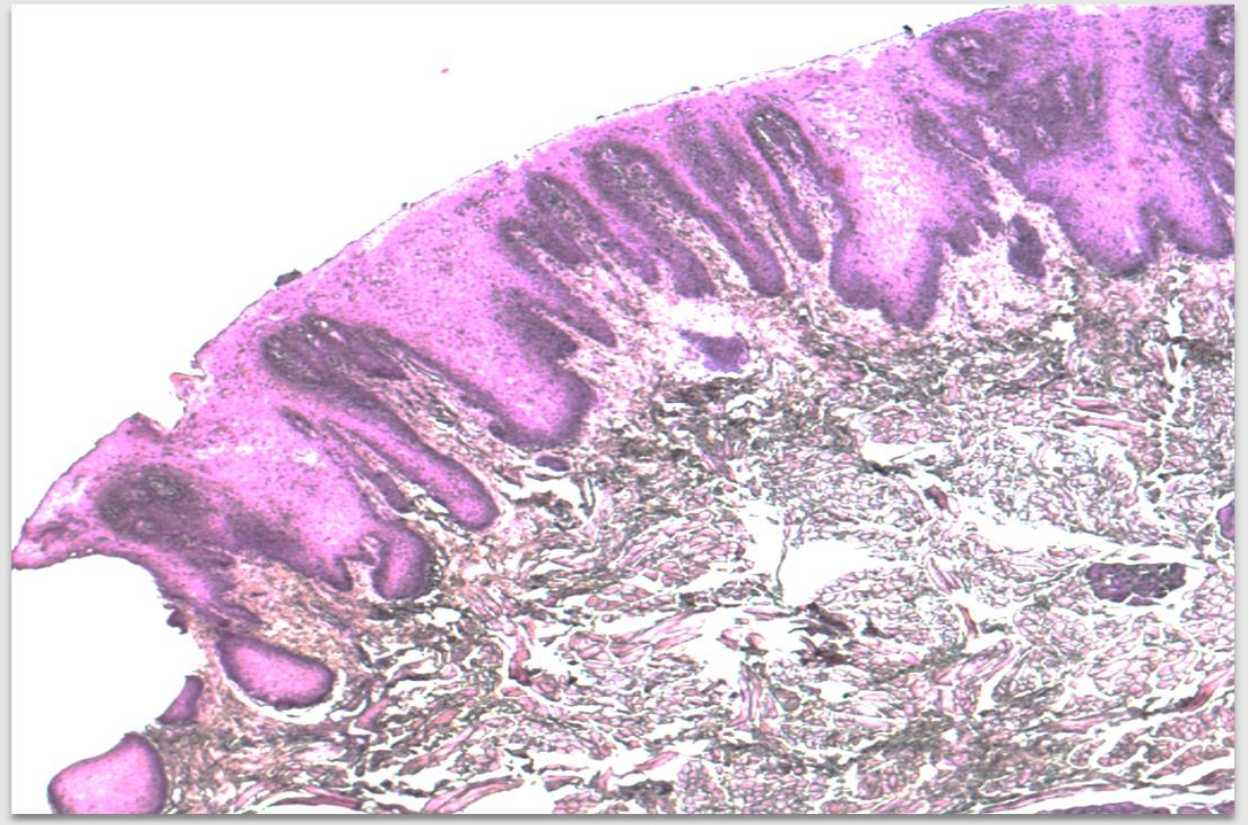

Figure 3b: **Histology slide of an old NHP (4X magnification)** thicker epithelial tissue layer; greater number of rete ridges compared to its younger counterpart in fig.3a

No differences are observed between the aged tissue sample slides treated with rapamycin (Fig. 3c) in terms of epithelial tissue thickness and number of distinct rete ridges when compared with the old NHP tissue slide (Fig. 3b)

Lipofuscin is a fine yellow-brown pigment granule composed of lipid-containing residues of lysosomal digestion.[25] It is known as one of the aging or "wear-and tear" pigments [44], found in body tissues which was also evaluated among the young, aged, and aged tissue samples treated with rapamycin.

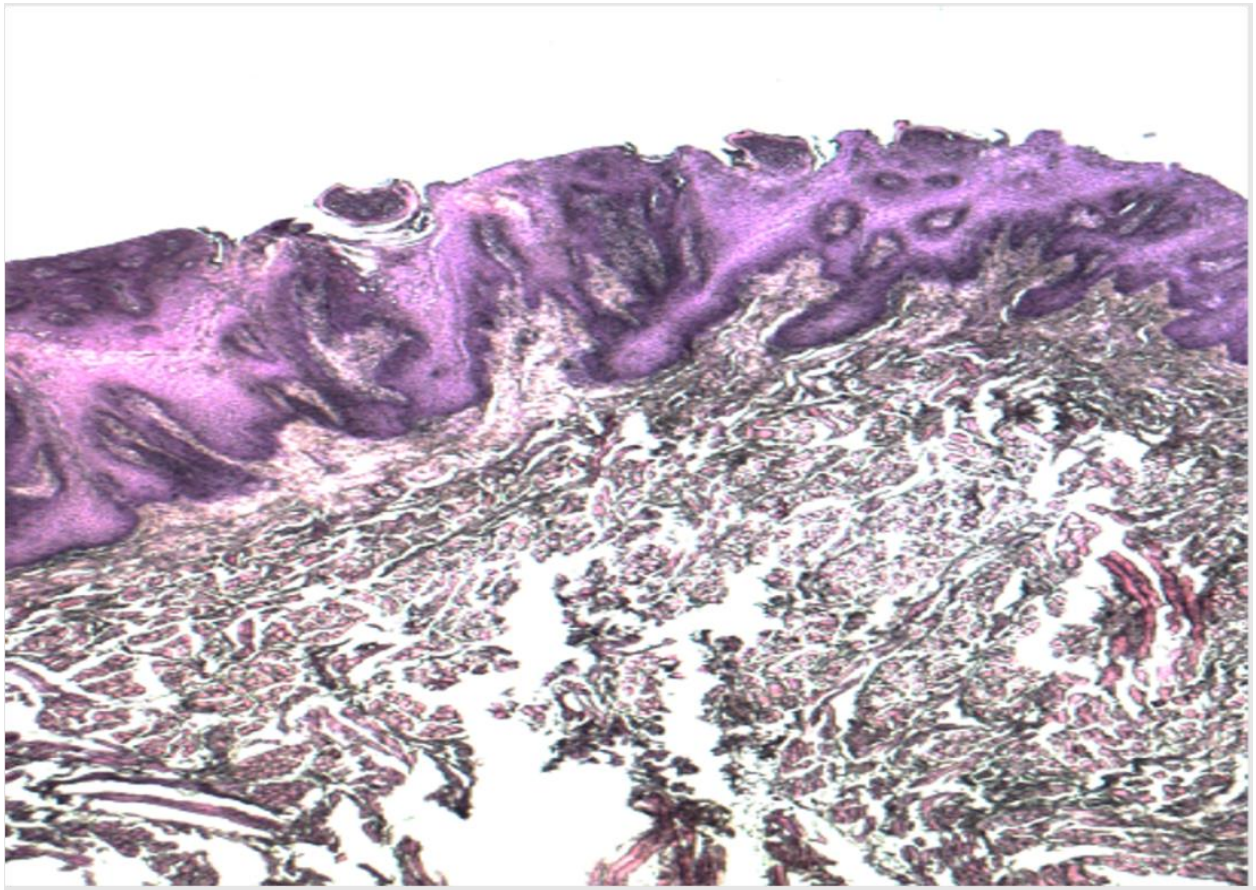

Figure 3c: **Histology slide of an old NHP treated with rapamycin (4X magnification)** thicker epithelial tissue layer; greater number of rete ridges compared to its younger counterpart in fig. 3a.

The presence of yellow brown pigment/lipofuscin granules was noted under 10X magnification in the young NHP tongue sample (Fig. 4a). However, lipofuscin granules are more visible and prominent in the old tissue sample slides (Fig. 4b).

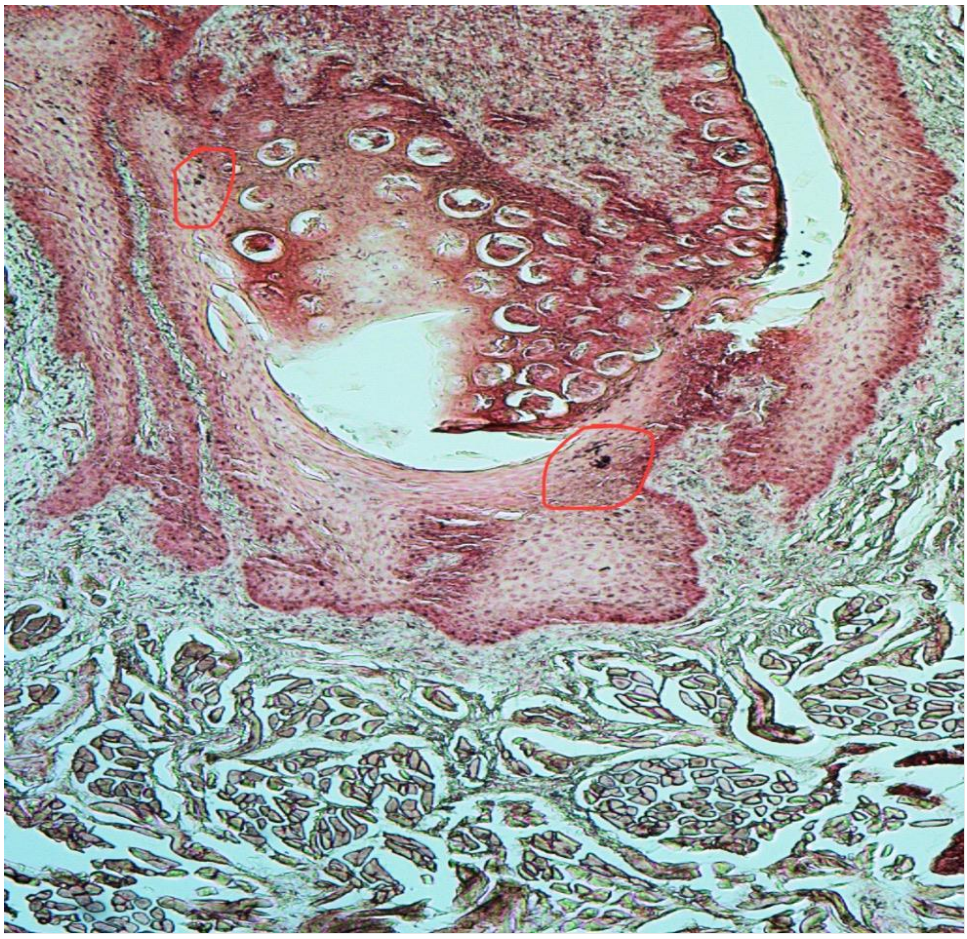

Figure 4a: **Histology slide showing lipofuscin granules in a young NHP (10X magnification)** circled in red.

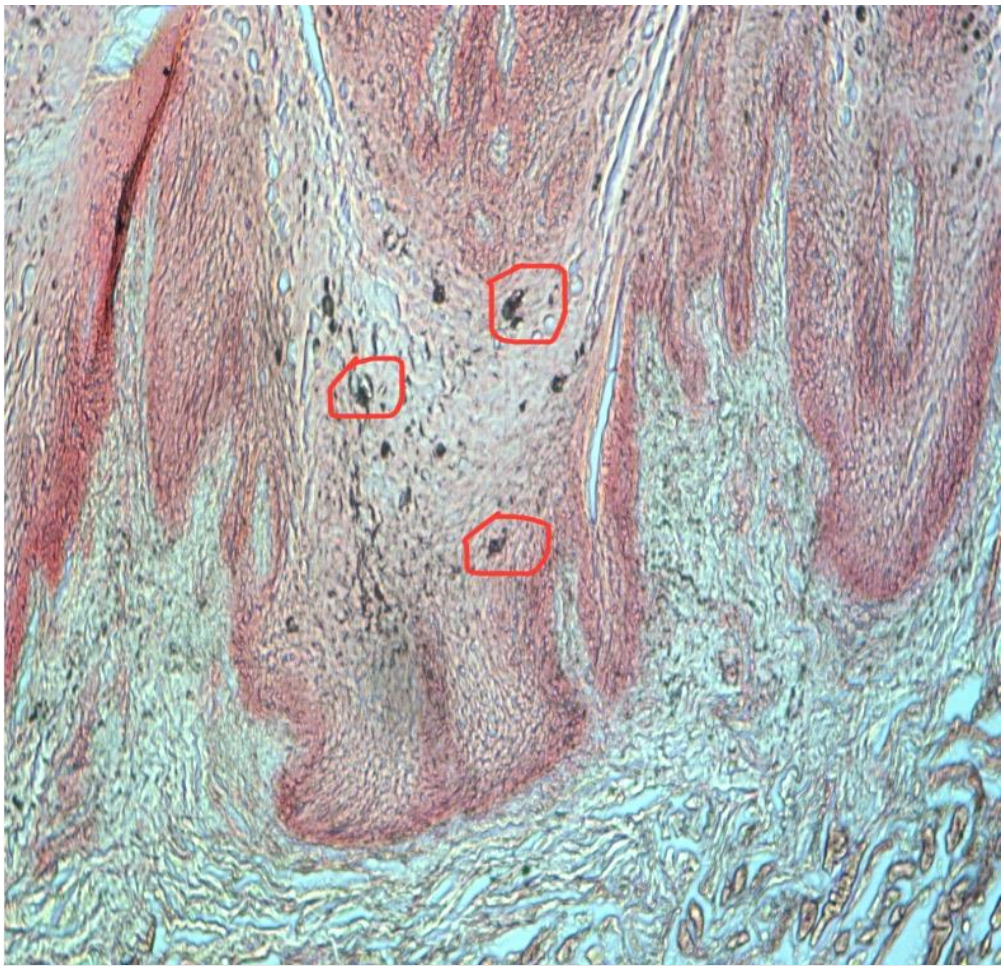

Figure 4b: **Histology slide showing lipofuscin granules in an old NHP (10X magnification)** increased presence of lipofuscin granules, circled in red.

There was no observable qualitative difference in the presence of lipofuscin granules seen in aged vs aged samples treated with rapamycin, when observed and compared under 20X magnification (Fig. 4c and 4d respectively).

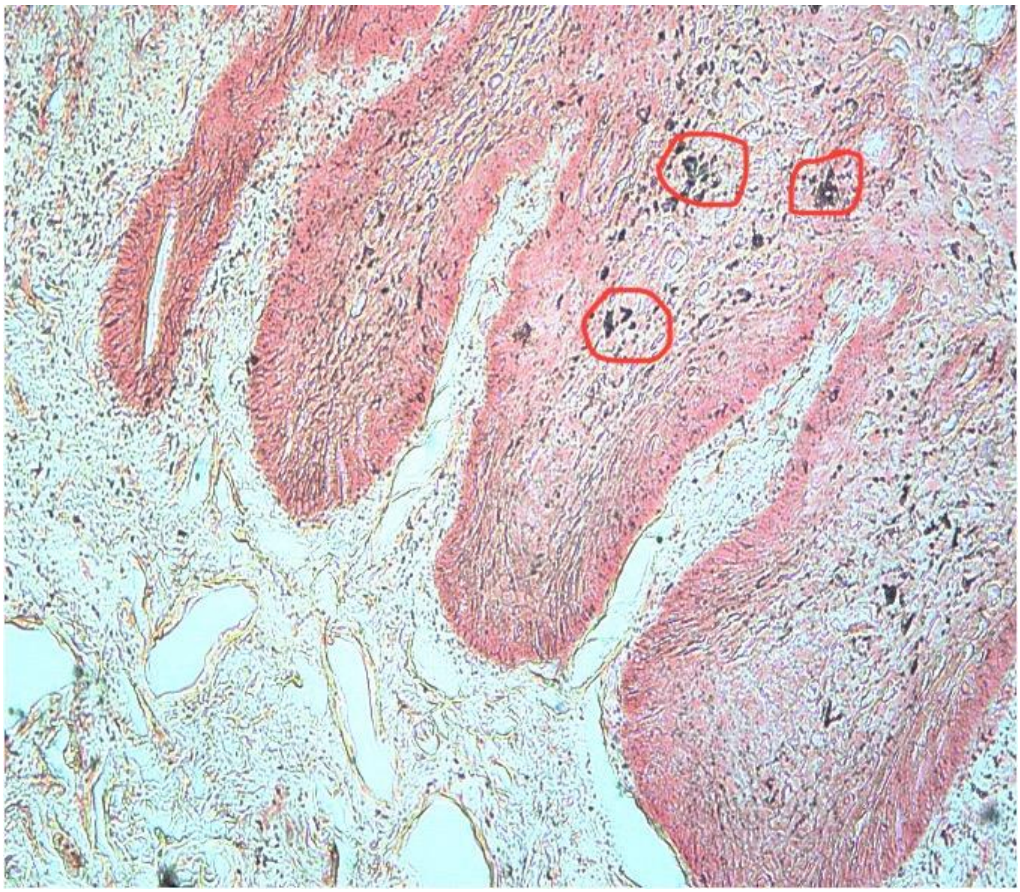

Figure 4c: **Histology slide showing lipofuscin granules in an old NHP (20X magnification)** circled in red.

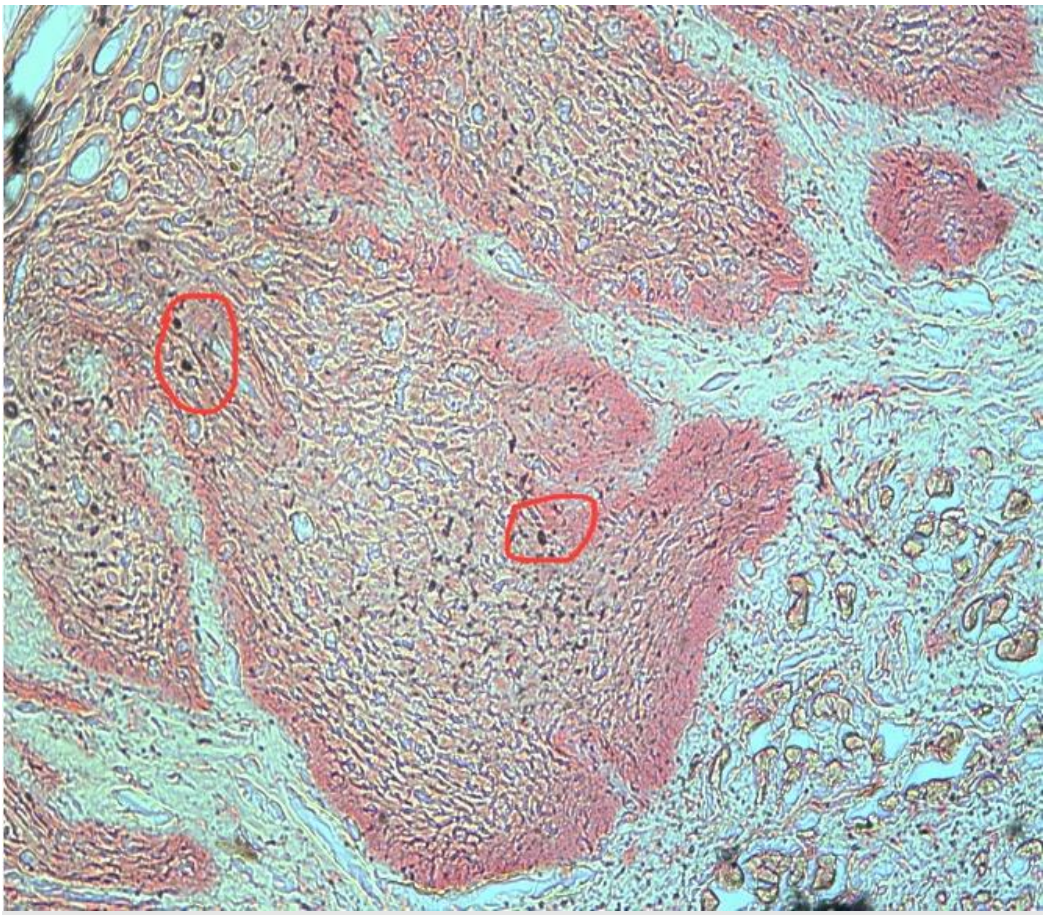

Figure 4d: **Histology slide showing lipofuscin granules in an old NHP treated with rapamycin (20X magnification)** circled in red.

V. DISCUSSION

This work is one of the first to examine cellular senescence in the healthy, aging tongue of nonhuman primates (NHPs), which shares a cross-species resemblance with humans. Studying cellular senescence captures the underlying changes brought on by the natural aging process that may provide future insights in understanding age-related diseases. We assessed two different senescence markers wherein our findings demonstrated that p21 expression in the epithelial tissues of aged NHPs was elevated when compared to that of the young, whereas the expression of p16 did not differ between the age groups. In addition, among the SASP markers, only IL1A was

significantly upregulated in the epithelial tissues of old NHPs compared to the young. In addition, rapamycin impacted the expression of various SASP factors but specifically decreased IL1A. Moreover, histological analysis of the tongue samples in the aged NHPs showed an increase in lipofuscin expression while also showing a thickened epithelium along with a greater number of rete ridges relative to the young NHP tongues. Together, the findings of this study provide initial evidence that the process of cellular senescence is occurring at the molecular level in the oral epithelial layer of the tongue with age.

Both p16 and p21 halt cell division, which can be upregulated in senescent cells, and can also induce senescence. [21,54,57] In our study, p21 was elevated in the epithelial tissue of the aged NHP compared to the young, while p16 showed no difference. A recent analysis of senescent cell markers in human tissues revealed an increase in both p21-positive and p16-positive cells with aging in the skin's epidermis and pancreas, while just the p16 was increased in the liver and intestine (colon) and p21 only in the skin's dermis. [47] Therefore, it is evident that different organs and tissue layers exhibit varying quantities of the senescent proteins p16 and p21 with age. While prior studies have demonstrated that p21 expression levels vary in oral squamous cell carcinoma (OSCC). [32,33] There were no clinical or histological signs of OSCC or other pathology in our aged tongue specimens. Hence, the elevated p21 expression is a function of normative aging and not pathology. This is in line with the previous literature, which has shown that with age, one or both markers (i.e., p16 or p21) will aid in identifying senescent cells.[48,53-56] The underlying cause for p21 elevation and no changes in p16 expression of the epithelial tissues in our study is unknown, but it is likely that distinct senescence pathways are present in the tongue, which may vary with age and pathology. Hence, future work could incorporate analyzing other areas of the tongue (apart

from the posterolateral region of the tongue that was isolated and examined in our study) and incorporating a larger set of samples of much older specimens.

Our study also demonstrated that rapamycin produced variable effects on the cell cycle arrest markers p16 and p21. While p16 increased with rapamycin treatment in both the epithelial and muscle tissue, p21 expression was increased in the epithelial tissue but decreased in the muscle. Studies by Sasaki et al. and Laberge et al. have shown that SASP markers could be downregulated by rapamycin without affecting p16 and p21 expression. [43,46] Moreover, it is now known rapamycin works in complex ways and its ability to affect cell cycle arrest markers (i.e., p16 and p21) depends on the biological activities within the cells. [45] Furthermore, p16 and p21 are found to be regulated independently of each other. [43]

Cell cycle arrest and the generation of SASP are two of the key processes that make up cell senescence. [45] Therefore, to better understand the role of p16 and p21 (cell cycle arrest markers) in the aging tongue, we evaluated inflammation, which is a critical component and associated with cellular senescence. [50] Prior research has demonstrated that long-term tissue inflammation and many age-related illnesses, such as cancer, are facilitated by the persistent release of inflammatory markers collectively called SASPs. [14,22] Our results demonstrated that only IL1A was upregulated in the epithelial tissue of old NHPs versus the young. This finding is in line with previous studies wherein IL1A expression increased in senescent cells. [23,51] IL1A is a pro-inflammatory mediator involved in regulating immune responses and inflammation. [38] However, the role of IL1A in disease is complex. For example, overexpression of IL1A in the epithelial cells of the colon mucosa is associated with driving inflammation and its related diseases (colitis) in these tissues. [38] On the other hand, IL1A can also stimulate immune response to protect the colon mucosa from infection. [64,65]

In cancer, it has been shown that low levels of IL1A at the local level can be protective [40,41] while overexpression at the systemic level can promote tumor progression. [66,67] However, the function of IL1A also depends on cancer type. [38] In our study, despite the increase in IL1A, we did not observe any clinical or histological evidence of cancer/disease in the older tongues. Further, we also observed that rapamycin was able to attenuate the increased IL1A expression. Our results are in line with past reports demonstrating rapamycin treatment downregulation of IL1A and the secretion of other inflammatory SASPs in senescent human fibroblasts. [43,52] Due to the absence of disease or pathology in the aged tongue, we are unable to conclude whether the increased IL1A expression during age is a protective effect or a precursor to pathology. What is certain is that rapamycin treatment decreased IL1A expression, and tongue specimens still showed no disease or pathology. Hence, while the combination of increased cell cycle arrest (p21) and IL1A indicates the increased cellular senescence of the aging tongue epithelial, whether this increases the risk for pathology or disease warrants additional samples and evaluation of much older animals.

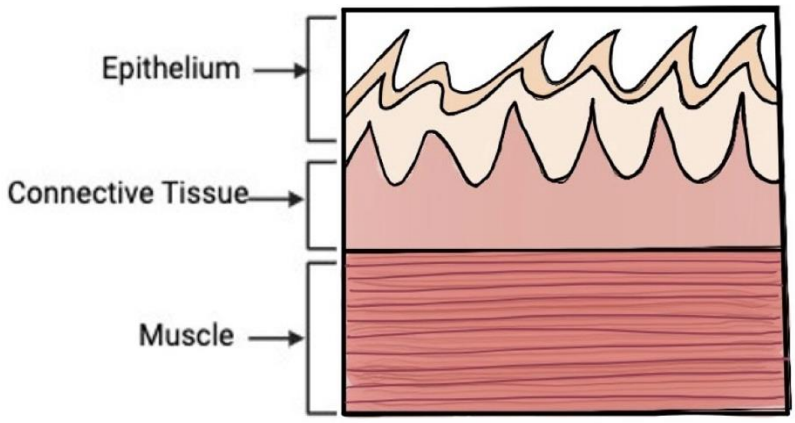

Fig.5: **Picture illustrating tongue epithelial tissue layers.**

In addition to evaluating the SASP markers, standard histology was completed to assess as well as compare different morphological features and investigate lipofuscin granules between our three study groups. Lipofuscin is a yellow-brown pigment which is a collection of oxidized proteins, lipids, and metals that is known to build up in aging tissues [25] and is considered a reliable hallmark of aging. [26] Our results demonstrate an increase in lipofuscin expression in the aged NHP tongue samples when compared to young, this result is in line with studies completed in the past. [27-29] Additionally, the older NHP samples showed thickened epithelium and an increased number of rete ridges. These findings however are in contrast with the past literature that demonstrates thinning of epithelium and blunting of rete ridges in the older mice (23 months ~66 human years) and humans (~60 plus years) across different oral sites selected. [30,31] In our study the "older animals" were in the range of 52-58 human equivalent years, thus evaluating even older animals (~75 human equivalent years) may provide different histological results.

Put together, our study is one of the first to investigate healthy tongues during normal aging using non-human primates. While we were able to look at multiple markers of senescence and the effects of rapamycin on the aging tongue there were a few limitations as well. There are additional senescent markers that could be investigated for an even more comprehensive picture of senescence in the tongue such as upregulation of cyclin D1 (an important regulator of cell cycle progression) [60,61] and senescence-associated beta-galactosidase (SA-β-gal or SABG). [62] Also, evaluation of male NHPs may provide a different view of the process of cellular senescence in the aging tongue.

VI. CONCLUSION

During age, NHP tongues show an increase in the cell cycle arrest marker p21, and a significant increase in IL1A expression. Moreover, rapamycin treatment blunted the increased IL1A expression levels. While more research is required to determine whether suppression of IL1A is advantageous or detrimental in the tongue during age, this study demonstrates that cellular senescence is ongoing during health rather than disease alone. Future research should focus on the role of cellular senescence in the risk of and development of various age-related oral conditions such as burning mouth disorder, and oral premalignant disorders.

CONFLICT OF INTEREST:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

VII. ACKNOWLEDGMENTS

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