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Efficacy Of Optical Coherence Tomography In The Detection Of Potentially Malignant Oral Mucosal Lesions Of The Tongue: An Animal Model

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APPROVAL PAGE

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Efficacy Of Optical Coherence Tomography In The Detection Of Potentially Malignant Oral Mucosal Lesions Of The Tongue: An Animal Model

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is the sixth most frequently occurring cancer worldwide. In the US approximately 50,000 new cases are diagnosed per year, representing 90% of all the cancer cases of the oral cavity. When oral squamous cell carcinoma is diagnosed it is often at an advanced stage. Novel technology to enhance early detection of oral squamous cell carcinoma could lead to early, less intensive treatments that increase patient survivorship as well reduce toxicity.

In recent years Optical Coherence Tomography (OCT), a new non-ionizing, non-invasive method, has been utilized to visualize and diagnose malignant lesions in diverse fields such as Ophthalmology, Gastroenterology, and Dermatology.

The objective of this current research was to demonstrate the efficacy of OCT for diagnosing potentially malignant oral mucosal lesions of the tongue. Forty nine mice were used in this study: 39 mice were treated with the carcinogen 4-nitroquinoline 1-oxide (4NQO) for up to 13 weeks, and 10 mice served as untreated controls. After specified treatment times, the posterior regions of the tongues were evaluated with the use of OCT and histopathologic analyses. Images of the histopathological sections were used as the gold standard, and compared with the OCT images to verify the accuracy of the OCT diagnoses.

Two examiners evaluated clinical images and OCT images of the 49 specimens, which were compared with the histological results. This comparison demonstrated that clinicians were more accurate in the clinical identification of

the normal aspect of the tongues, however some differences were found in the results of the clinical evaluation of the treated group with the histological results.

The analyses of control group OCT images compared with the histological results were moderately accurate as were those obtained with the clinical evaluation. However, the comparison of results of the evaluation with the OCT of the treated group and the histological analysis were different between the examiners and were not correctly classified based on the histological results.

Comparison of these results demonstrated that clinical evaluation is important in the identification of possible lesions in the oral cavity, however the criteria and results may vary between dentists. OCT may become a useful imaging technique, in which images resembling the microstructural changes occurring in the epithelium of the oral cavity will guide the specialist to the precise area to be evaluated with the histopathological analysis. This study demonstrated differences between both examiners and variations in their consistency to evaluate the OCT images. Despite that OCT is a high resolution imaging technique in which images are similar to a low power microscope (4x), it is important that the clinician is familiar with the normal appearance and changes expected to be present in possible lesions that will be visualized in the OCT images and histological sections, to avoid false positive or false negatives.

ORAL CANCER

One of the major public health problems in the United States and many other countries of the world is cancer; approximately one in four deaths in the United States is related to cancer [1]. Oral cancer is the eighth most-common cancer among white males and the sixth most common cancer among Afro-American males in the United States. In other regions of the world, especially in South-Central Asia, head and neck cancers are the most common malignancies found in men. In the U.S., approximately 9,000 deaths per year are a consequence of oral cancer, making it more deadly than breast cancer, cervical cancer and prostate cancer. It has been estimated that one person is killed, every hour, every day by oral cancer [2, 3].

Oral cancer is defined as any cancerous tissue growth located in the mouth. It may arise as a primary lesion originating in any of the oral tissues or by metastasis from a distant site of origin. Squamous cell carcinoma, which develops from the stratified squamous epithelium that lines the mouth and pharynx, is the most common cancer diagnosed of all the different types of cancer that may be found in the oral cavity [4-6].

Squamous Cell Carcinoma (SCC) of the oral region is the sixth most common malignancy worldwide [4]. Approximately 50,000 new cases of squamous cell carcinoma of the head and neck are diagnosed each year in the United States. According to the American Cancer Society, in 2013 the total estimated number of new cases of cancer in all anatomic sites was 1,660,290, and 41,380 was the estimated number of new cases of oral and pharynx cancer, representing 2.5%

of all the new cases of all sites in the body [7]. Cancers of the lips, tongue, floor of the mouth, palate, gingiva, alveolar mucosa, buccal mucosa, and oropharynx will account for approximately 30,000 of these cases [5]. According to Jemal et al. 48 % of all cancers located in the head and neck are located in the oral cavity and 90% of these are OSCC [8]. In the past, squamous cell carcinoma of the oral cavity was primarily found in male patients aged 60 years and older with an extensive history of tobacco smoking, alcohol consumption and poor oral hygiene, which may act additively or synergistically on a genetically susceptible individual. However, some studies have shown increased incidence of oral SCC (OSCC) among young patients, under 40 years of age, being more frequent in women with no history of tobacco smoking. Other agents that may contribute to the rise in the number of cases of oral cancer are various forms of drug abuse, environmental factors, diet and the human papilloma virus (HPV), although the HPV is not considered to be a significant risk factor of the anterior two thirds of the tongue or the remaining oral cavity [3, 9-12].

Approximately 50-55 percent of patients with oral cancer survive beyond five years; this rate has not improved during the last 50 years. When the oral cancer is localized at diagnosis the five year survival rate is approximately 75%, but in general this disease is diagnosed in stages 3 and 4 with lymph node metastasis, reducing the probability of five-year survival to 26.5% [3, 12, 13].

The tongue is the most common site for oral cancer in both American men and women. This is also true of developed countries, however, in some developing countries, site prevalences may differ owing to culture-specific habits, for

example, nasopharyngeal cancer in Southeast Asia and buccal cancer in India are the most common oral and pharyngeal sites [5]. Patients with oral tongue squamous cell carcinoma have a significantly worse prognosis than those with similar lesions of the oropharynx, larynx, hypopharynx and other sites of the oral cavity [4]. The tongue includes a rich lymphatic network and a complex structure of muscles that make it the site most frequently associated with cervical metastasis compared to cancer of other sites in the oral cavity [10].

Other common areas of oral cancer are: floor of the mouth, lip mucosa, retromolar gingiva, hard palate, buccal mucosa, lower and upper alveolar ridge. But cancers that develop in the tongue, floor of the mouth and lip mucosa represent more than 70% of all oral cancers [11].

POTENTIALLY MALIGNANT LESIONS

At an early stage tongue OSCC is typically asymptomatic, making early diagnosis challenging [14]. Therefore, clinical evaluation and possibly soft tissue imaging are the key methods for early identification and diagnosis of oral tongue squamous cell carcinoma. It is vitally important to recognize clinical signs and symptoms suggestive of oral squamous cell carcinoma at an early stage. These include oral ulceration (non-healing), raised, everted, exophytic and indurated lesions, abnormal swellings, loss of tongue mobility, dysarthria, otalgia, cauliflower-like or warty growths, abnormal localized tooth mobility, non-healing tooth sockets, color changes in mucosa (red, white or speckled patches), erosive, raw mucosal patches, reduced or altered orofacial sensation [15]. One of the greatest challenges that the dentist has is the dilemma to predict which of these lesions will progress to neoplasia, like the most frequent in the oral cavity, oral squamous cell carcinoma. Nevertheless, the biological relevance of benign and malignant lesions may not be distinguishable on the basis of their clinical appearance [12, 16].

Dentists must be alert to the presence of subtle lesions that could be considered as premalignant and early stage malignant lesions. The World Health Organization defines premalignant lesions as morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart. The most common lesions to be considered as potentially malignant lesions in the oral cavity are oral leukoplakia and oral erythroplakia. Other lesions to be considered as potentially malignant are reverse smoker’s palate, oral submucous

fibrosis and tobacco pouch keratosis [16, 17]. Clinicians should focus their attention on high risk sites where 90% of premalignant lesions arise; these are the floor of the mouth, ventrolateral aspect of tongue and soft palate [18].

Leukoplakia is defined as a white patch or plaque that cannot be characterized clinically or pathologically as any other disease; the most common sites are buccal mucosa, alveolar mucosa and lower lip. Lesions that arise on the floor of the mouth, lateral tongue and lower lip are most likely to show dysplastic or malignant changes and are considered high risk sites [17]. Any leukoplakia that persists more than 10-14 days after conservative treatment should be considered as a potentially premalignant condition [19].

Erythroplakia is defined as lesions of the oral mucosa that present as red areas and cannot be diagnosed as any other definable lesion. This lesion is uncommon compared with leukoplakia, and is found predominantly in middle aged and elderly people, with predilection for soft palate, buccal mucosa, tonsillar pillars and floor of the mouth. Some differences in location were found depending on gender. In men the most commonly affected site is the floor of the mouth, followed by the retromolar trigone, while in women erythroplakia is more common in the mandibular alveolar mucosa, mandibular gingiva and mandibular vestibule. Oral erythroplakia has the highest risk for malignant transformation compared with other premalignant lesions [17, 19].

DIAGNOSTIC METHODS

Dentists utilize a variety of aids to diagnose oral precancerous and cancerous lesions. As with any test, proper case selection and correct performance of the test itself are critical to the sensitivity and specificity of its result. Some of these aids are: brush cytology (brush biopsy), tissue fluorescence, and toluidine blue staining. Some techniques utilized by specialists are: punch biopsy, scalpel biopsy, fine-needle aspiration cytology and sentinel node biopsy [19].

Imaging techniques used to detect squamous cell carcinoma include plain radiography, computed tomography, magnetic resonance imaging and molecular imaging [18, 20, 21]. These imaging techniques are used to define the borders of the affected tissues (bone or soft tissue), the presence of internal contents in the lesion, regional lymphatic spread and the effects on adjacent tissues (expansion, remodeling, displacement, destruction). OSCC is difficult to diagnose in an early stage because the malignant changes are confined to the soft tissues. Most oral lesions are benign, but many have an appearance that may be confused with a malignant lesion, and some previously considered benign are now classified as premalignant because they have been statistically correlated with subsequent cancerous changes. Conversely, some malignant lesions seen in an early stage may be mistaken for a benign change. Any oral lesion that does not regress spontaneously or respond to the usual therapeutic measures should be considered potentially malignant until histologically shown to be benign [20, 22].

Recently, Optical Coherence Tomography (OCT) has been implemented in dermatology and oncology to determine the presence of soft tissue lesions [23].

OCT is a noninvasive diagnostic imaging modality with a high resolution that can give near histologic images with a safe broadband light source. This imaging technique measures backscattered light generated from an infrared light source directed at the tissues being examined [24]. Broadband laser light waves are emitted from a source and directed toward a beam splitter. One wave is sent toward a reference mirror with a known path length and the other toward the tissue sample. After the 2 beams reflect off the reference mirror and the tissue surfaces at varying depths in the sample, the reflected light is directed back toward the beam splitter, where the waves are recombined and read with a photo detector [25] (Fig. 1). OCT is the optical equivalent of ultrasound, using light instead of sound waves; it is a noninvasive and nondestructive method for imaging the microstructural detail of oral tissue *in situ.* Resolution up to 1-2 μm can be achieved, being 100-250 times greater than high-resolution ultrasound [26]. It is capable of evaluating the health of hard and soft tissue by providing a cross-sectional “optical biopsy” of tissue up to 3 mm in depth from the surface. The resultant images have an axial resolution of 1–10 μm, capturing structural details without the use of ionizing radiation and not possible with conventional x-ray imaging technologies [27]. OCT is considered to be an optical “biopsy” by some authors because the image resembles the architecture observed in histology [26, 27]. For this technique to become clinically interpretable and relevant, the structures visualized must be correlated with the corresponding tissue microstructures [28].

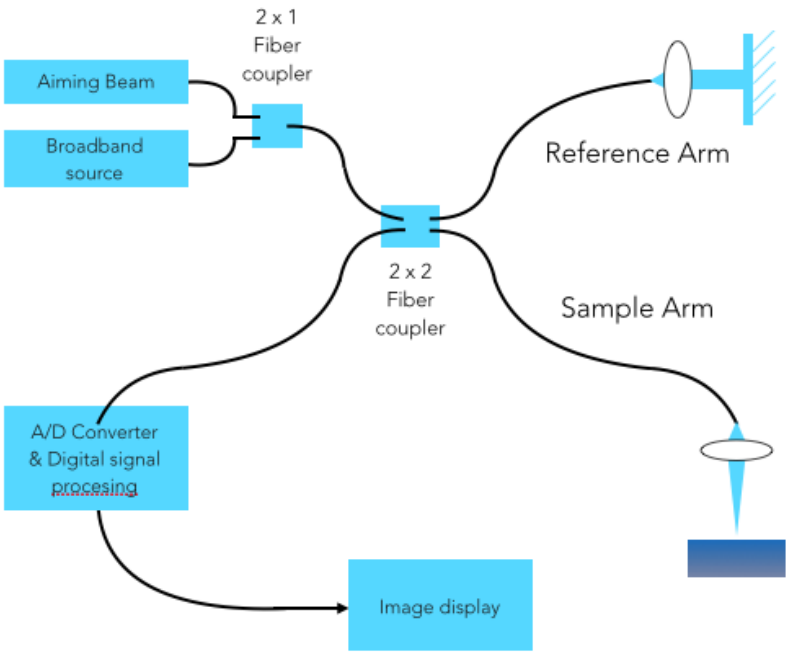


Fig. 1 Diagram of the Optical Coherence Tomography based on:
http://www.answers.com/topic/optical-coherence-tomography and
In Vivo Optical Coherence Tomography for the Diagnosis of Oral Malignancy, Wilder-Smith et al, Lasers in Surgery and Medicine 35:269–275 (2004)

The resolution of OCT permits *in vivo* noninvasive imaging of the macroscopic characteristics of epithelial and sub-epithelial structures including: depth and thickness, peripheral margins, and potential histopathological appearance [29]. OCT has been used to detect soft tissue malignant lesions in the oral cavity in a small number of studies but less frequently than with other areas such as ophthalmology, gastroenterology, urology and gynecology [30-33]. Current identification and diagnosis of potentially malignant and malignant oral mucosal lesions rely upon histologic and cytologic examination performed by a pathologist after suspicious tissue is biopsied. These methods represent the gold standard for cancer diagnosis. However, they have some limitations, including the invasive nature of the biopsy, cost of the procedure, and typically two-three days

from time of biopsy to obtaining the histopathologic results. Importantly, diagnostic interpretation of the tissue sample may vary among pathologists [29, 34]. Additionally OSCC may not be detected until clinically advanced; subtle oral mucosal lesions may be overlooked by the clinician during visual inspection of the tissue [35]. Early malignant changes are still overlooked using conventional oral examination; an example of this is dysplasia that may be found in “clinically normal mucosa” [36]. A recent meta-analysis reported 93% sensitivity for conventional oral examination, while specificity was only 31% [36]. Recently some devices such as VELscope [36, 37], Identafi [36, 38], and Narrow Band Imaging (NBI) [36, 39], have been used to improve the evaluation of the oral mucosa to identify potential malignant lesions. Nevertheless, the differentiation of low and high risk lesions using some of these devices remains undetermined, with false positives obtained in some cases. These devices may affect the lesion’s appearance in terms of brightness, texture and delineation of the margins but they have not been shown to enhance the ability to identify potentially malignant lesions, especially those that are not visible under normal operatory lighting. Early-stage lesions often are asymptomatic and may mimic other conditions, whereas others may not be readily evident in routine examination. Also, because malignant and benign lesions may not be clinically distinguishable, the clinician cannot predict the biological relevance of lesions on the basis of their physical features alone [12].

OBJECTIVES

The objective of this study is to demonstrate the efficacy of Optical Coherence Tomography to diagnose potentially malignant oral mucosal lesions of the tongue.

HYPOTHESIS

OCT image findings in potentially malignant oral mucosal lesions of the tongue are comparable to histopathologic findings.

SPECIFIC AIMS

1) To induce potential oral tongue SCC in mice using the carcinogen 4-nitroquinoline 1-oxide;

2) Obtain OCT images of the tongue lesions and compare them with histopathological sections of the lesions; and

3) Determine quantitatively the correlation between OCT and histopathological images for specific structural parameters.

MATERIALS AND METHODS

Study Design:

All experimental procedures were performed at the University of Connecticut Health Center in complete concordance with the guidelines of an approved protocol (ACC# 100776-1016) for animal experimentation and in compliance with the guidelines set forth in the *Guide for Care and Use of Laboratory Animals*. Forty nine female C57BL/6J mice, 7 weeks-old, were used for this study, 10 as a control group and 39 with induced oral tongue squamous cell carcinoma. A total of fifty mice were received to start this project, but the first day of the experiment one mouse died, finishing with a total of 49 mice. The animals were acquired from Jackson Laboratory, Bar Harbor, Maine, United States of America. The OCT instrument that was used for this study was donated by Axsun Technologies, Inc. (Axsun Technologies, Inc., Billerica, MA) to the Section of Oral and Maxillofacial Radiology of the School of Dental Medicine, University of Connecticut. This device is used for research specifically in the area of dentistry.

Inducing Potentially Malignant Lesions in the Oral Cavity

After 6 days of acclimation to their surroundings and a 12-hour light/dark cycle, mice were randomly placed in 1 of 10 groups, each group with a total of 5 mice except one group that had 4 mice. The first two groups with ten mice in total comprised the control group and the remaining 39 mice were treated with the carcinogen agent (See experimental timeline, Fig. 2.). The mice were maintained

on standard rodent chow and water *ad libitum*, under normal laboratory conditions in the Center for Comparative Medicine, UCHC, in Room LB033C.

The first day of the experiment, a solution consisting of distilled water with 100 p.p.m. 4-nitroquinoline 1-oxide (4NQO) was provided to the test group to chemically induce potentially malignant mucosal lesions of the tongue. The 4NQO is water-soluble and can be administered orally in drinking water. (The 4NQO was acquired through Sigma- Aldrich, St. Louis, MO.) A fresh batch of water was prepared every week for each of the first to thirteenth week of carcinogen treatment. The mice drank this water with dilute 4NQO for up to thirteen successive weeks. Control mice received water without any additive. The level in the water bottles was monitored two times per week.

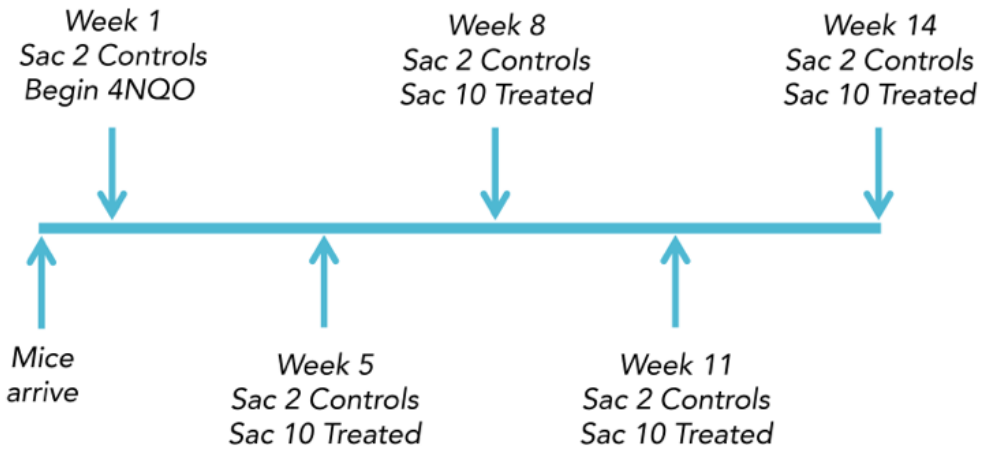


Figure 2: Experimental Timeline

Tanaka states that this carcinogenic agent produces all the stages of oral carcinogenesis with similar histological appearance and molecular changes that are observed in the human system [40]. The 4NQO is a synthetic carcinogen derivative of a quinoline, is soluble in water and sensitive to high temperature

and light (the 4NQO solution was stored in a brown glass bottle and brown glass water bottles were used with the mouse cages). One of the advantages of this carcinogen agent is that it is capable of inducing sequentially the different phases of carcinogenesis (hyperplasia, dysplasia, severe dysplasia, carcinoma *in situ* and OSSC) [41]. The lesions obtained with the use of the 4NQO appear similar to damage imposed by other carcinogens present in tobacco, which is a major risk factor for oral cancer [42].

The 4NQO is a powerful carcinogen that may act in several organs, and it can specifically induce tongue SCC when it is applied in low concentrations *via* drinking water [43]. This potent mutagen and carcinogen, after metabolic activation, binds to DNA producing three main adducts: two on guanine (dGuo-N2-AQO, dGuo-C8-AQO) and one on adenine (dAdo-N6-AQO) [44]. The 4NQO induces histological as well as molecular changes similar to those observed in human oral carcinogenesis. 4NQO has been used for the induction of oral cancer including dorsal and ventral tongue, palate and aerodigestive tract.

Wellness Monitoring and Euthanasia

Depending on the group to which the mice were randomly assigned, they were exposed to 4NQO added to the drinking water or no treatment at all. In order to monitor the food and water intake, and the appearance and activity of the mice during the experiment, the mice were observed twice per week and weighed once per week, and these data were recorded in an Excel spreadsheet. In general, the mice gained or maintained their weight; a few treated mice lost a

small amount of weight, not more than 10% of body weight (between 0.5-1 g), over the full course of the experiment. Although both groups gained weight during the course of the experiment, the mice in the treated group did not gain as much as the ones in the control group. When the mice arrived at the Center for Comparative Medicine at UCHC, the mean weight was 18.70 g. Their weight increased progressively until the 14th week, when the experiment finished; the mean weight of the two last mice of the control group was 23.95 g compared to the mean of the last 10 mice of the treated group of 20.40 g (Fig. 3).

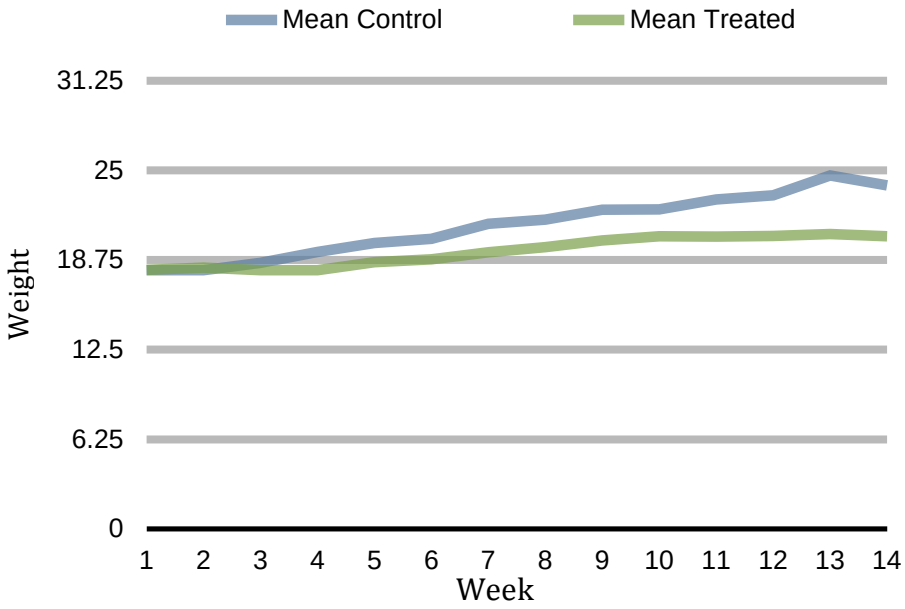


Fig. 3 Comparison of the body weight for the mice in control group vs Treated group

The first day of the experiment two mice of the control group were euthanized by CO_2 inhalation. Subsequently, starting on the fourth week and every three weeks thereafter, 2 mice of the control group and 10 of the treated group were sacrificed using the same method of CO_2

inhalation followed by cardiac transsection. The last 2 control mice and the last 10 treated mice were euthanized on the fourteenth week.

Obtaining OCT Images and Histological Sections:

After euthanasia, the tongues of the mice were excised and placed in a container with cold Phosphate Buffered Saline (PBS) identified with the number of the respective specimen. In general the location of the excision was at the posterior aspect of the throat.

After excision of the tongues a clinical evaluation was performed to evaluate any macroscopic changes, the tongues were photographed using a Canon camera EOS 60D with a Canon 28-135 IS lens, and the images were saved as RAW images.

The posterior region of each tongue was scanned, using the OCT (Axsun Technologies, Inc), as many times as necessary to completely scan the area of its dorsum and ventral aspect. The images obtained from the scan were saved as TIFF images. To standardize the scan procedure, the tongues were placed in the middle portion of a round plastic device, the scanner was positioned in a perpendicular position to the table where the specimen was located; the same procedure was used to scan all 49 tongues. Once the tongues were scanned using the OCT they were fixed in 10% buffered formalin.

The fixed tongues were sent to the Section of Oral and Maxillofacial Pathology to proceed with a histological study of each specimen. The tongues were embedded in paraffin and the histological sections were stained with hematoxylin and eosin and coverslipped. The sections were scanned into the Aperio virtual

microscope system in the Faculty Instructional Technology Service, UCHC, using either the 20X or 40X objective lens. The sections were histologically evaluated in the same orientation as the OCT was previously performed.

Image Analysis:

Images obtained by OCT were coded and analyzed by a radiologist at the Section of Oral and Maxillofacial Radiology at the University of Connecticut School of Dental Medicine. The Section of Oral and Maxillofacial Pathology provided histopathologic evaluations of the tongue sections. The criteria used for the evaluations were based on the histological changes visualized in the different sections, such as: hyperkeratinization, changes of the filiform papillae, increased thickness of the stratified squamous epithelium and changes of the basement membrane. According to these, the sections were categorized as: Normal, Hyperkeratosis, Hyperkeratosis with maturational disturbance and Maturational disturbance. A radiologist and pathologist evaluated the clinical images of the different tongues. The criteria to evaluate the tongues were based on the clinical appearance, color of the dorsum and ventral aspect of the tongues and the presence of any potential lesion such as leukoplakia or erythroplakia. According to these, the tongues were classified as: Normal, that included all tongues with pink appearance, smooth surface and no lesions on their dorsal, ventral or lateral aspects, possible lesion was used when the evaluators considered that the tongues did not have a normal appearance but were not sure of the presence of an entity affecting the tongues, and the final category was lesion, that included

changes of the appearance of the tongue and presence of probable hyperkeratosis or other lesions such as leukoplakia or erythroplakia.

The OCT images of the posterior tongue were evaluated using the following criteria: changes in the keratinized layer, changes in the thickness of the keratinized layer, in the thickness of the stratified squamous epithelial layer and changes observed in the basement membrane. The areas to be evaluated were the posterior, middle and tip of each tongue. According to the changes that were visualized the tongues were classified as Normal, when no changes were noted, possible lesion, when some changes were noted but the evaluators were not sure of the presence of a lesion, and the final option was lesion, that included significant changes noted by the evaluators (Fig. 4). The evaluation of the OCT images was repeated by the same evaluators one week after the first evaluation was done.

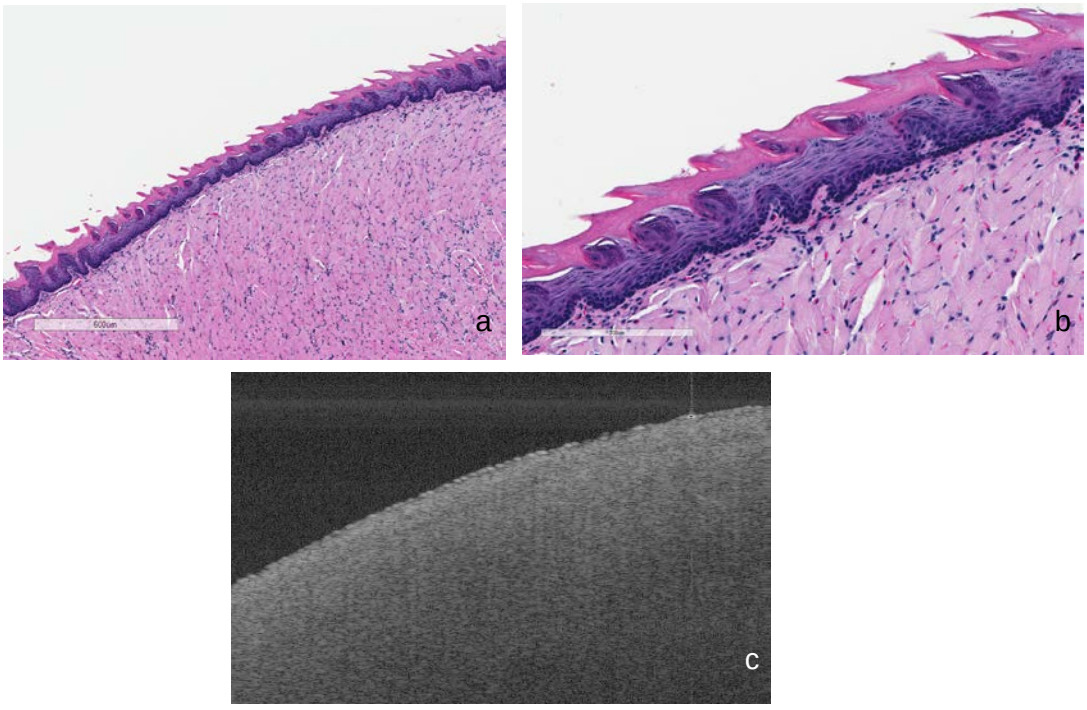


Fig. 4 Histological (panels a and b) and OCT images (panel c) of the middle portion of normal mouse tongue. Scale bars = 600 μm in (a) and 200 μm in (b).

The clinical images and the OCT images were presented in a Keynote slide show (Apple, Cupertino, CA). The first four slides showed the normal aspect of the tongues, clinically and OCT images as well; after these four slides, the different clinical images and OCT sections were presented in a random order. For the second evaluation of the OCT images the order was changed to avoid bias with the previous results. The results obtained in both the clinical and OCT evaluations were compared with the histological classification determined by an oral pathologist, which was used as the gold standard.

RESULTS

All the mice except one were included in the study. The one animal excluded from analysis died on the first day of the experiment. All mice included in the study remained in apparent healthy condition and had a slight increase in body weight (Fig. 3).

Clinical evaluation

Forty nine mice were used in this experiment, 10 of these were randomly selected for the control group and the remaining 39 mice were included in the treated group. The majority of the clinical changes noted in the treated group occurred on the dorsum of the tongue with the exception of one specimen that presented a verrucous appearing lesion on its ventral aspect (Fig. 5).

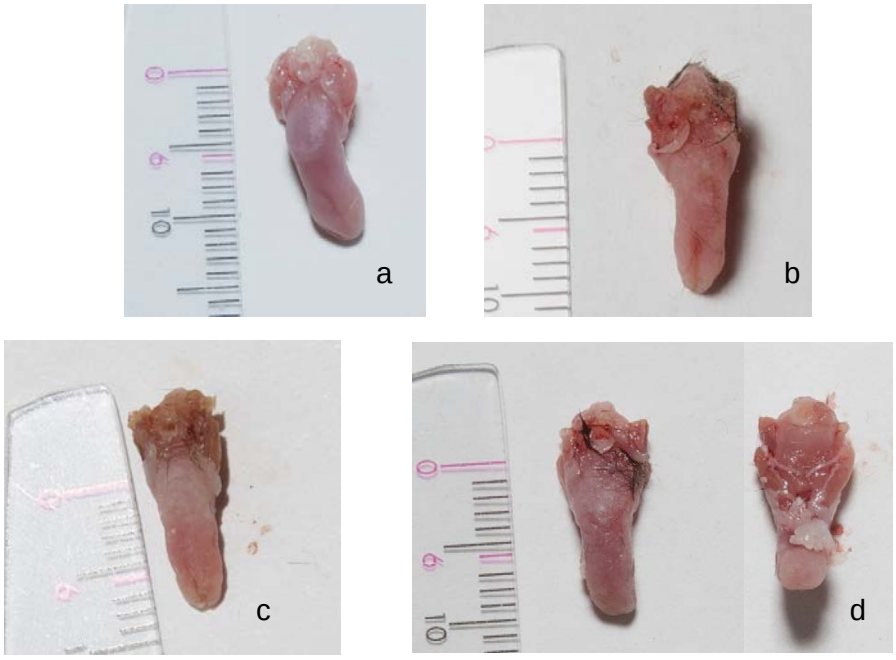


Fig 5. Comparison of clinical images: a) Normal appearance of mice with no treatment sacrificed in week 14 . Fig. b,c and d. Tongues of mice receiving 4NQO dissolved in drinking water and sacrificed at weeks 8, 11 and 14, respectively. Note the verrucous lesion in ventral aspect of the tongue (d).

During the clinical evaluation of the untreated tongues, evaluator 1 classified 8 of these as normal and 2 with a possible lesion, while evaluator 2 considered that 6 of 10 were normal, 3 with a possible lesion and 1 was classified with a lesion (Fig. 6).

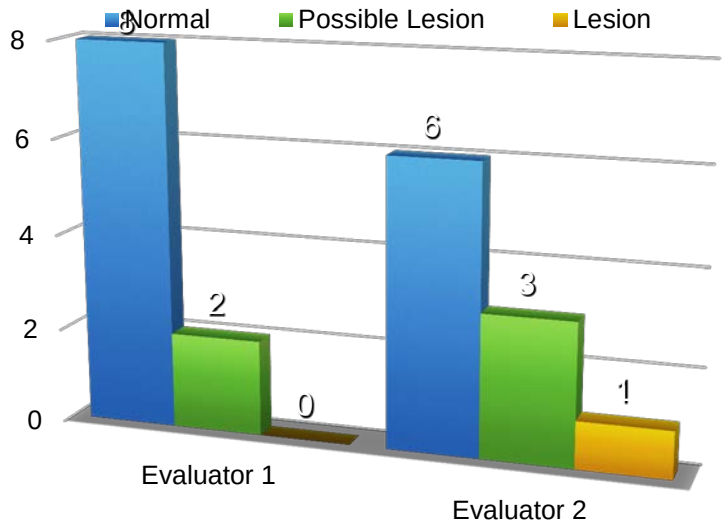


Fig. 6 Clinical evaluation of Control group

,These results were compared with the histological results in order to determine the accuracy of the clinical evaluation. This accuracy in the evaluation of the normal group was 80% for evaluator 1 and 60% for evaluator 2, but 20% - 40% of the specimens were categorized as possible lesion or lesion by the two evaluators. For this evaluation they did not have the opportunity to evaluate the tongues grossly, but only by observing images.

The most substantial difference in the clinical evaluation was found in the treated group. Evaluator 1 classified 18 as normal tongues while 21 were classified with possible lesions. The results of evaluator 2 contrasted with those of the first evaluator in that only 2 tongues were classified as normal while 12 were classified as possible lesion and 25 of the 39 specimens were categorized as having lesions (Fig. 7).

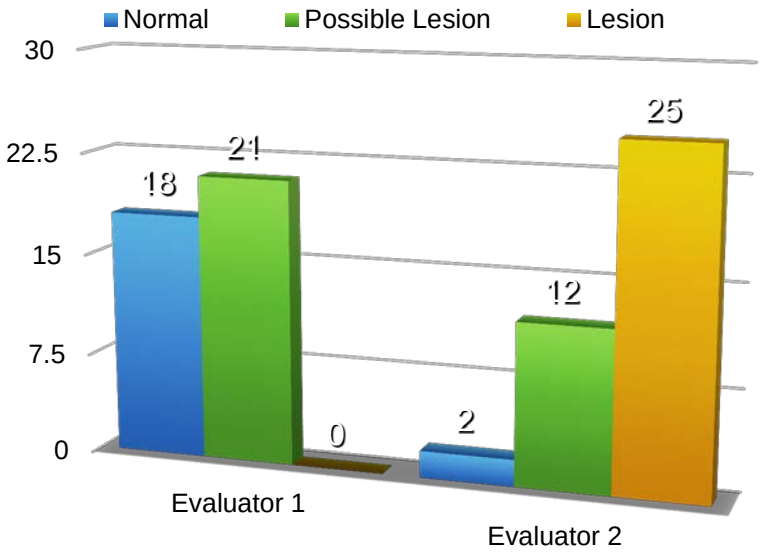


Fig. 7 Clinical evaluation of Treated group

The accuracy of this evaluation differed from the one obtained in the control group, because more specimens were classified as having possible lesions and lesions. For this reason, two comparisons were made, the first evaluation between the normal vs. lesions and the second corresponding to the evaluation of normal vs. possible lesions. Both evaluations were compared to the obtained histological sections.

Histologic results demonstrated that 9 specimens of the treated group had no histological changes and were classified as normal, while 30 specimens presented some variations such as Hyperkeratosis, Hyperplasia with normal maturation, Hyperkeratosis with maturational disturbance and Maturational disturbance; all of these categories were grouped as lesion.

Evaluator 1 was accurate in the identification of 3 treated tongues of 9 that were histologically diagnosed as normal while evaluator 2 identified correctly only 1 of the 9 tongues histologically classified as normal, but correctly identified 22 of 30 as tongues with lesions (Fig. 8).

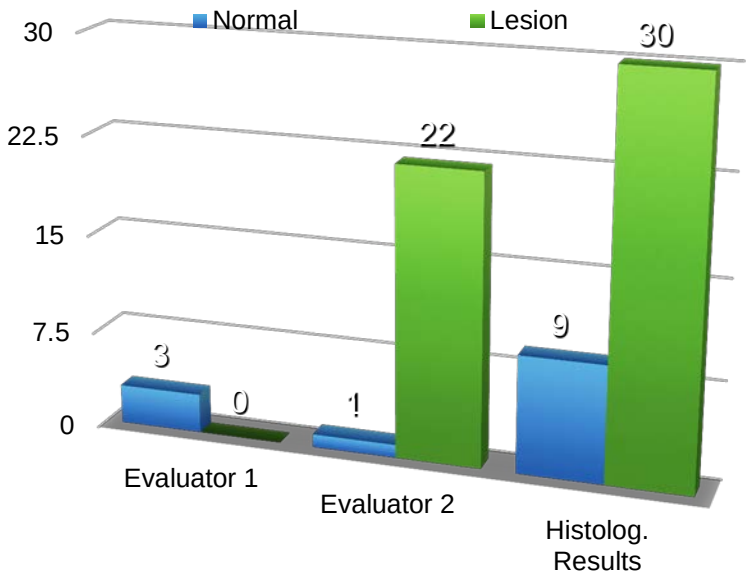


Fig. 8 Clinical Evaluation- Histological results, Normal vs Lesion

The results of the evaluation of the specimens that were categorized by the evaluators as possible lesions were compared to the tongues histologically diagnosed with lesions. For evaluator 1, 15 tongues were selected as having a

possible lesion of a total of 30 that were histologically diagnosed with some changes that were considered as lesions, while evaluator 2 identified 7 tongues as having possible lesions. Both evaluators used the category “possible lesion” when they considered that the tongues were not normal but did not exhibit enough changes to be considered as tongues with lesion (Fig. 9).

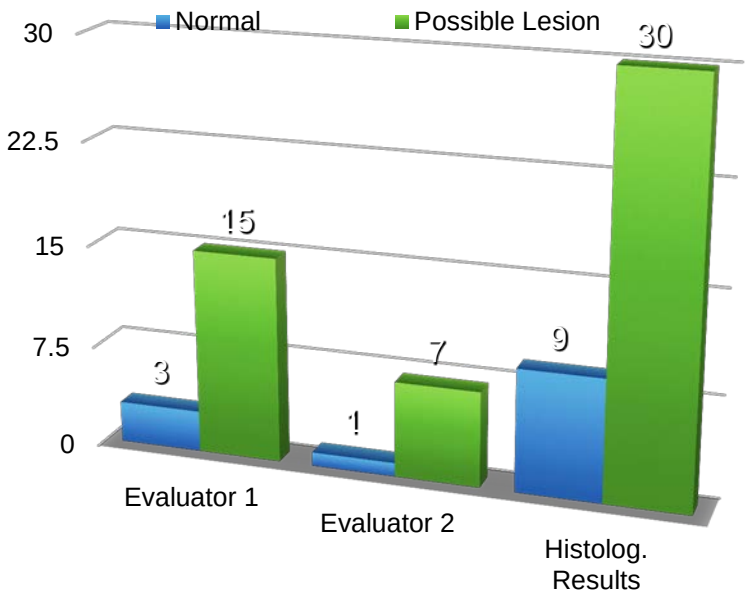
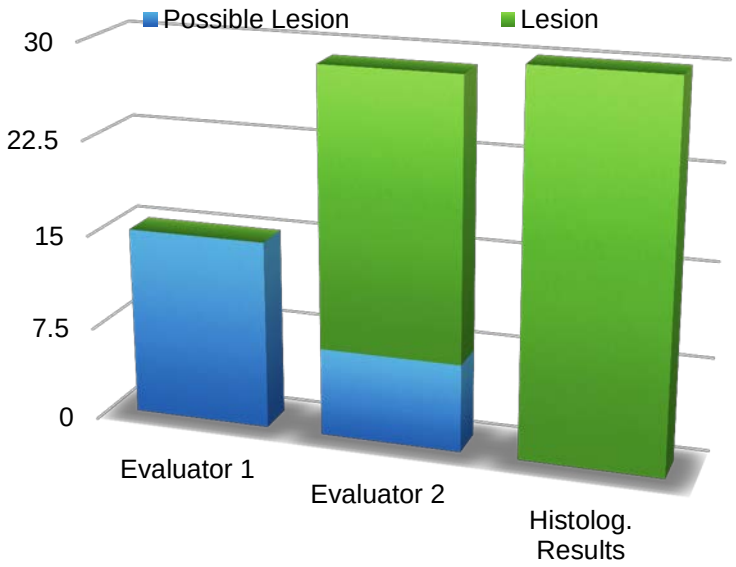


Fig. 9 Clinical evaluation- Histological results, Normal vs Possible Lesion

For the accuracy of the clinical evaluation of the treated group, evaluator 1 correctly identified 30% of the tongues as normal while 50% of the tongues histologically diagnosed with lesions were clinically identified as having possible lesions. Evaluator 2 correctly identified 1 specimen as normal, representing 11% of the diagnoses of the normal tongues. However, 29 tongues were identified as



having possible lesions or lesions, representing 97% of the tongues with lesions (Fig. 10).

OCT evaluation

The results obtained from the two evaluations made by the two examiners were compared to the histological results obtained from the Department of Oral Pathology of the University of Connecticut Health Center. No histologic changes were noted in the control group as was expected, however only 30 specimens of the treated group showed some histological changes such as hyperkeratosis, Hyperkeratosis with maturational disturbance and only maturational disturbance.

Fig. 10 Clinical evaluation- Histological results, Possible Lesion + Lesion

The most common changes noted in the specimens were hyperkeratosis followed by maturational disturbance (Figs. 11- 15).

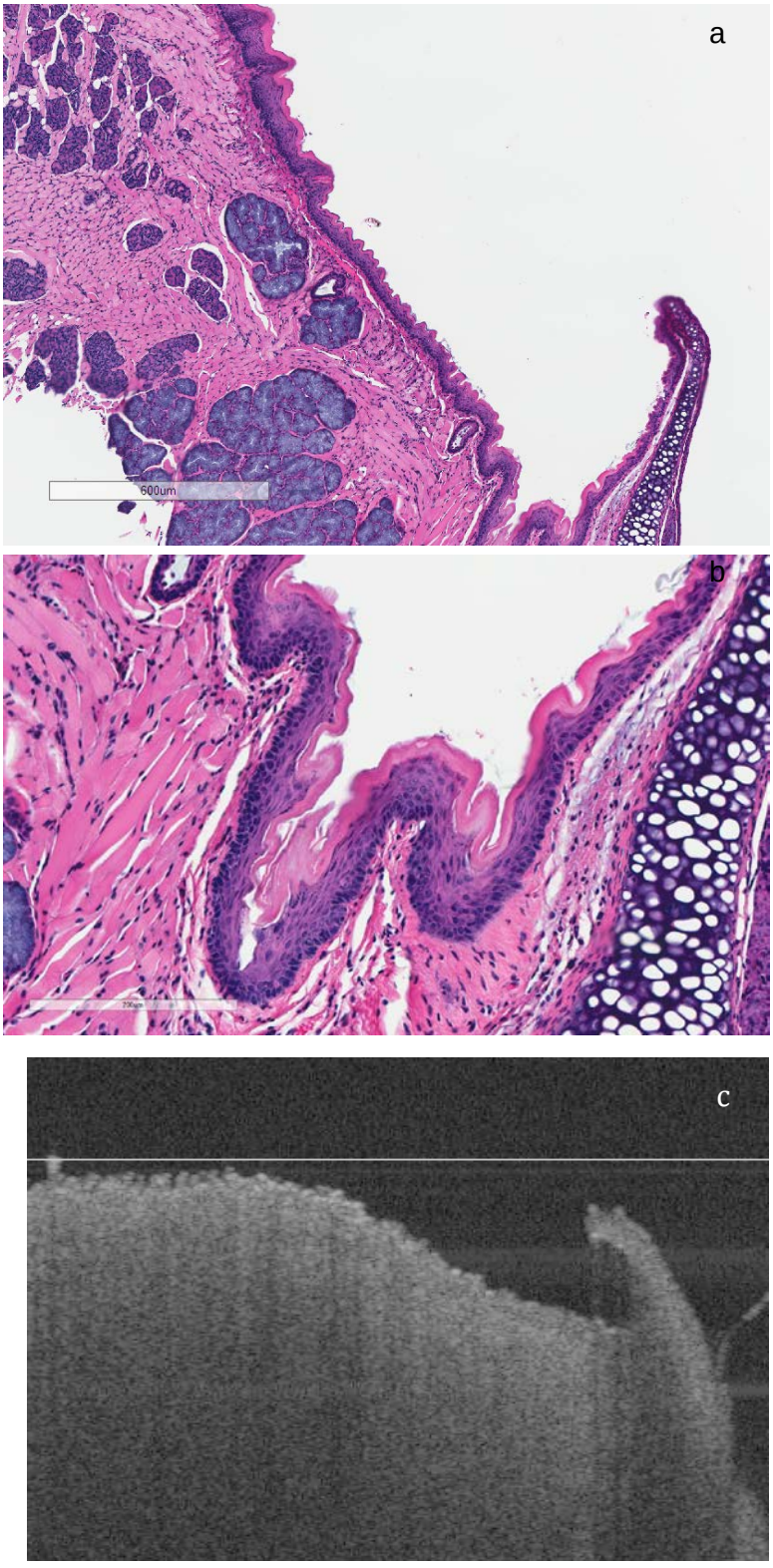


Fig. 11. Histological (Panels a and b) and OCT(Panel c) images of the posterior aspect of a mouse tongue with hyperkeratosis (sacrificed on the 5th week of the experiment) Scale bars = 600 μm in (a) and 200 μm in (b).

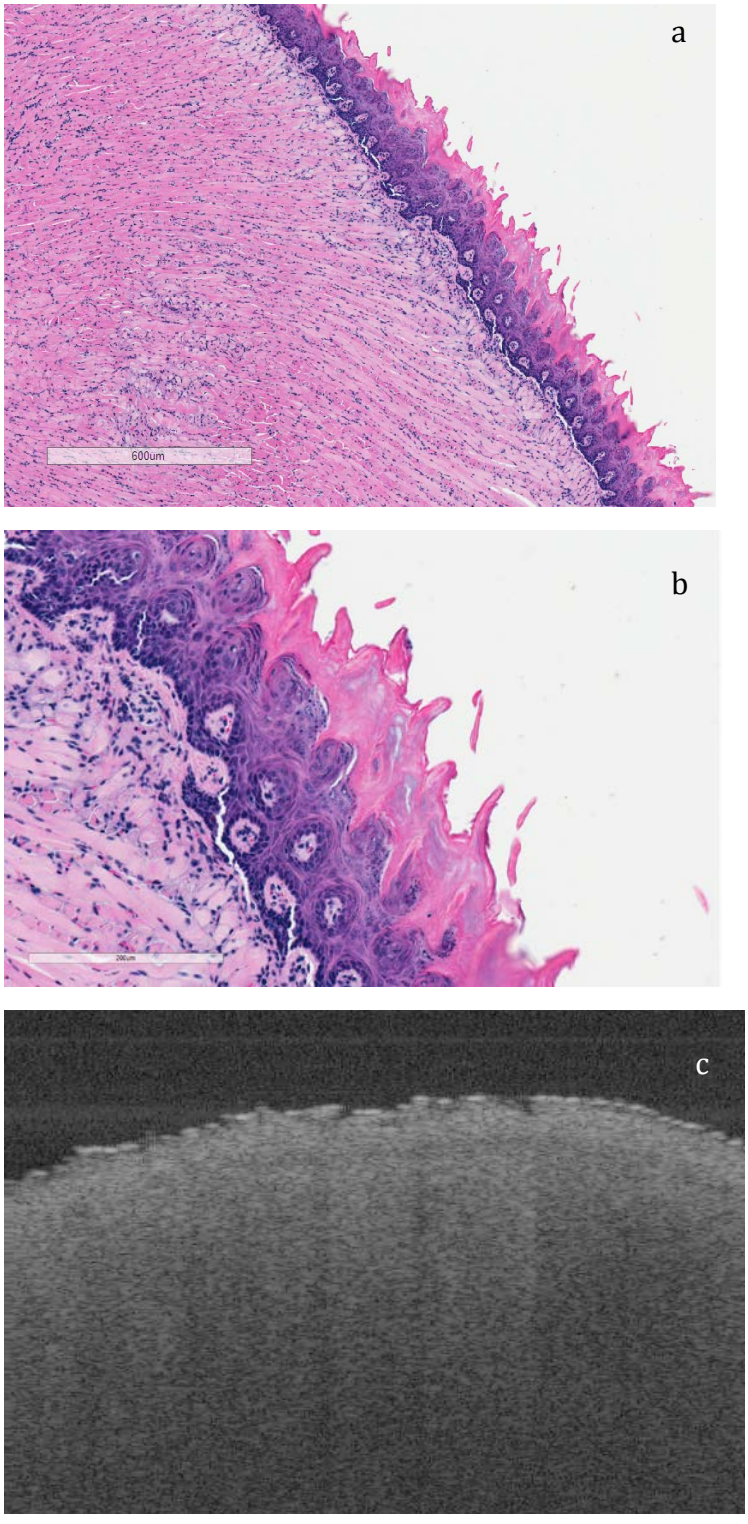
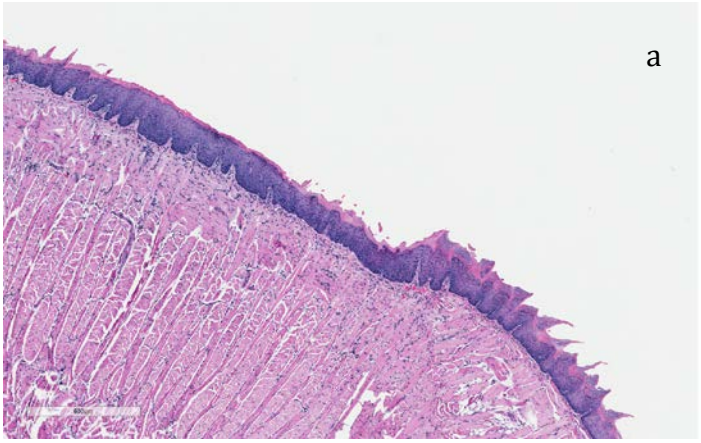
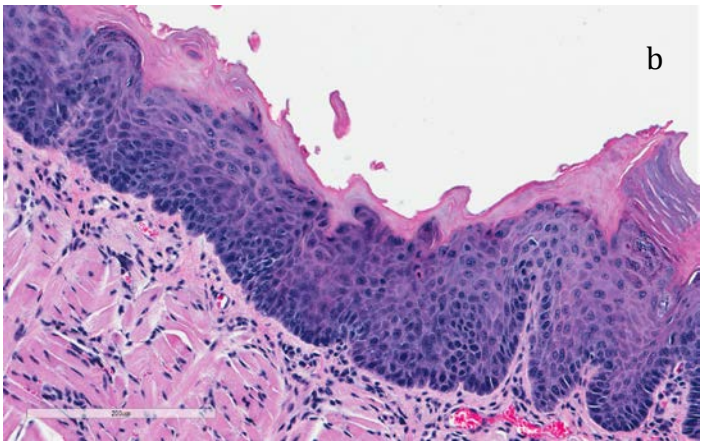
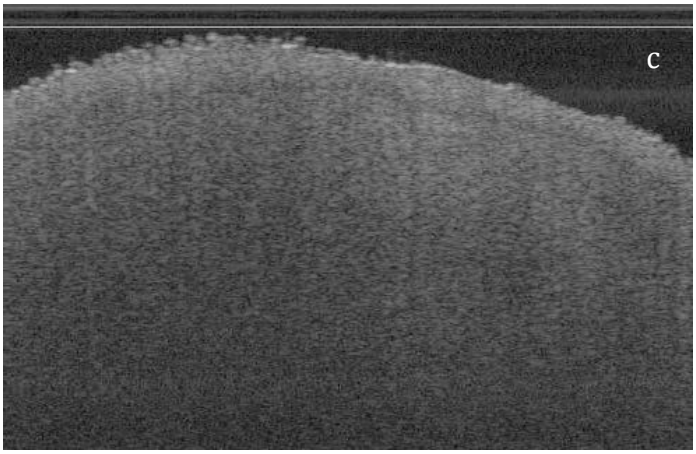
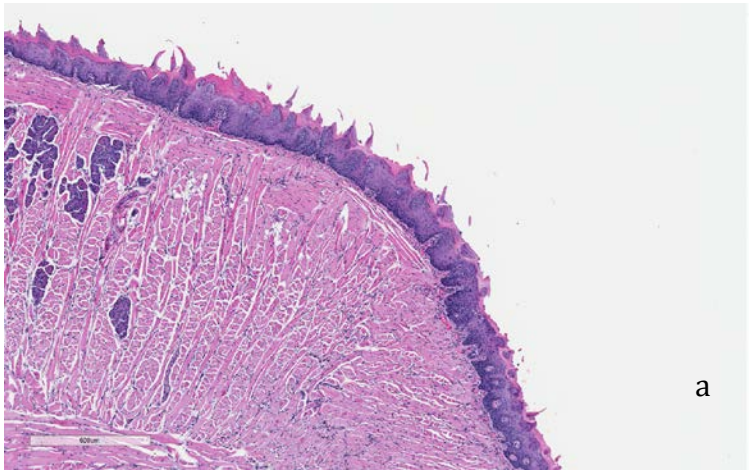


Fig. 12 Histological (Panels a and b) and OCT(Panel c) images of the middle aspect of a mouse tongue with hyperkeratosis (sacrificed on the 8th week of the experiment) Scale bars = 600 μm in (a) and 200 μm in (b).


a


b


c



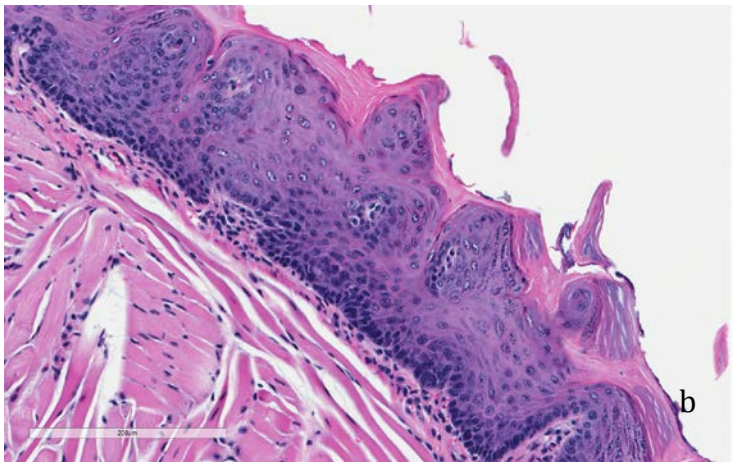


Fig.13 Histological (Panels a and b) and OCT(Panel c) images of the middle aspect of a mouse tongue with crowding in the stratum spinosum (sacrificed on the 11th week of the experiment) Scale bars = 600 μm in (a) and 200 μm in (b).

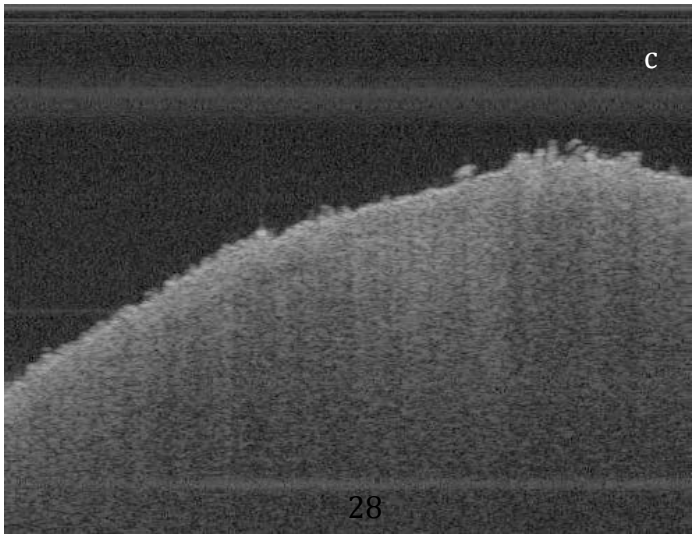


Fig. 14 Histological (Panels a and b) and OCT(Panel c) images of the middle aspect of a mouse tongue with pleomorphism in the stratum spinosum (sacrificed on the 14 th week of the experiment) Scale bars = 600 μm in (a) and 200 μm in (b).

a

b

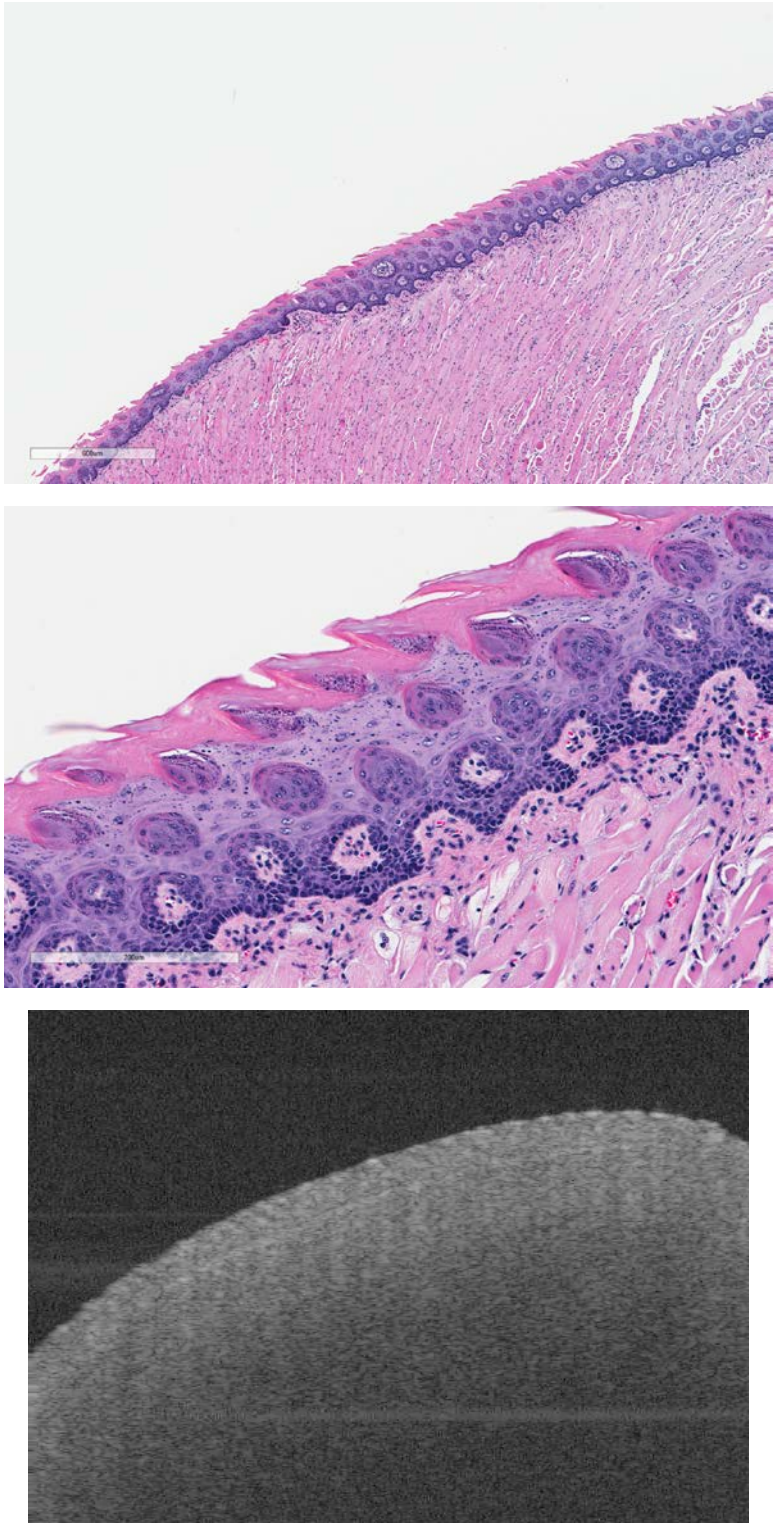


Fig. 15 Histological image of the middle portion of a normal mouse tongue (sacrificed on the 14th week)
Scale bars = 600 μm in (a) and 200 μm in (b).

The evaluation of the OCT images showed a marked difference between the two evaluators. A week after the clinical evaluation was done, the evaluators classified the OCT images; these images were presented in a 15” screen. All the images were randomly inserted in a Keynote slideshow. The first four slides showed the OCT images with the histological sections of the same site; the remainder of the slideshow did not contain any histological sections. The order of the image presentation in the slideshow was different to avoid bias in the evaluations.

The results obtained from Evaluator 1 for the two OCT evaluations of the control tongues were: in the first evaluation 10 of 10 were identified as normal and in the second evaluation only 9 tongues were accurately identified as normal while 1 tongue was categorized as having a lesion. Evaluator two identified 7 tongues as normal, 2 as tongues with possible lesions and 1 with a lesion, while in the second evaluation 8 tongues were classified as normal and 2 as having possible lesions. The accuracy for the evaluation of the OCT images compared to the histological results was very high for both evaluators. For evaluator 1 the accuracy rate was 90-100% while for evaluator 2 the accuracy rate was 70-80% (Fig. 16).

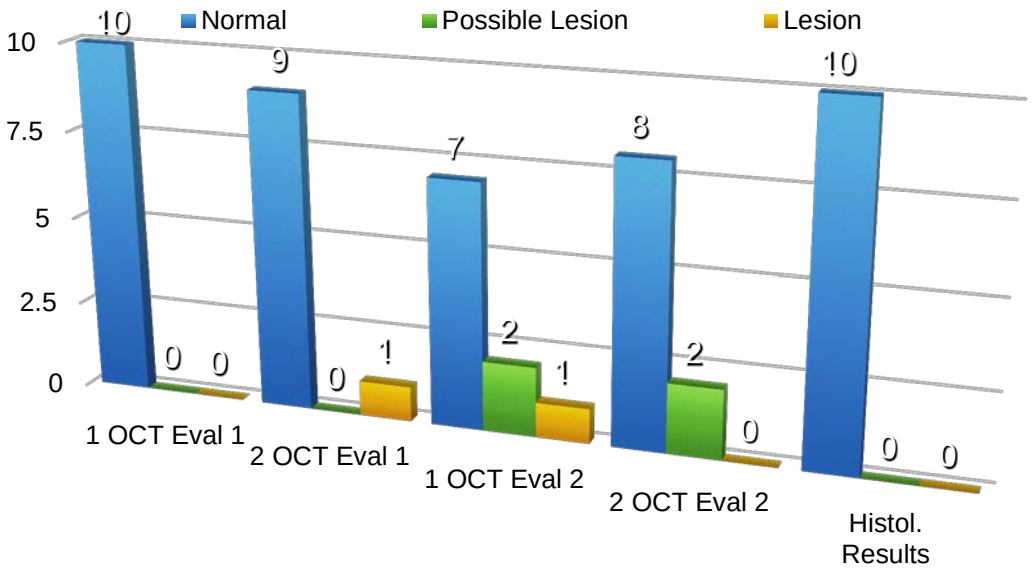


Fig. 16 Evaluation of OCT images and histological results of control group

For the first evaluation of the tongues of the treated group, Evaluator 1 classified 36 of the 39 tongues that were treated with 4NQO as normal, with no changes, while 3 were classified as having possible lesions. On the other hand, Evaluator 2 considered that only 8 of the tongues of the treated group were normal and had no changes in the epithelium, while 18 were classified as having possible lesions and 13 had lesions.

A week later the OCT evaluation was repeated, changing the order of the slides, and evaluating the same parameters. Evaluator 1 considered that 36 tongues had no changes and only 3 could have possible lesions. However, Evaluator 2 classified 10 tongues as normal, 12 as having possible lesions and 17 were included in the group of tongues with lesions. According to the histological

evaluation 9 of 39 treated tongues had no histological changes and the rest had some changes and were classified as lesions (Fig. 17).

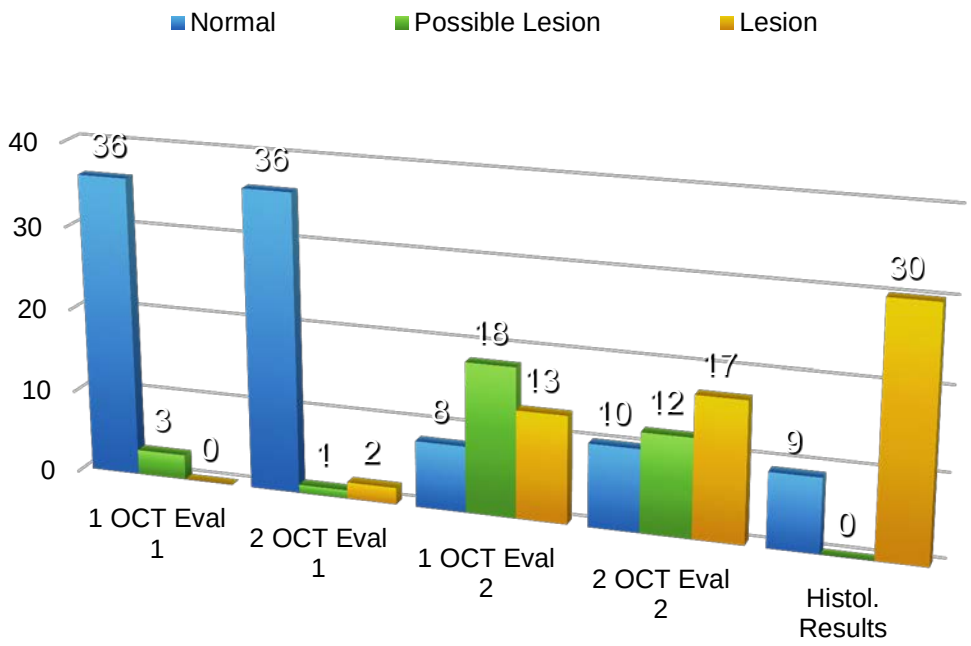


Fig 17. Evaluation of OCT images- Histological results, Treated group

The accuracy of the results obtained in the evaluation of the treated group differs from the evaluation of the control group. Evaluator 1 was accurate in the identification of the tongues that had no changes. In the first evaluation 8 tongues were correctly identified and for the second evaluation 7 were selected as normal, however, no tongues were classified as tongues with lesions in both evaluations. Evaluator 2 identified 2 of the 9 tongues histologically diagnosed with no changes and in the second evaluation only 4 were identified as normal.

However, 10 tongues were correctly identified as having lesions in the first evaluation while 15 of the 30 tongues with lesions were identified in the second evaluation (Fig. 18).

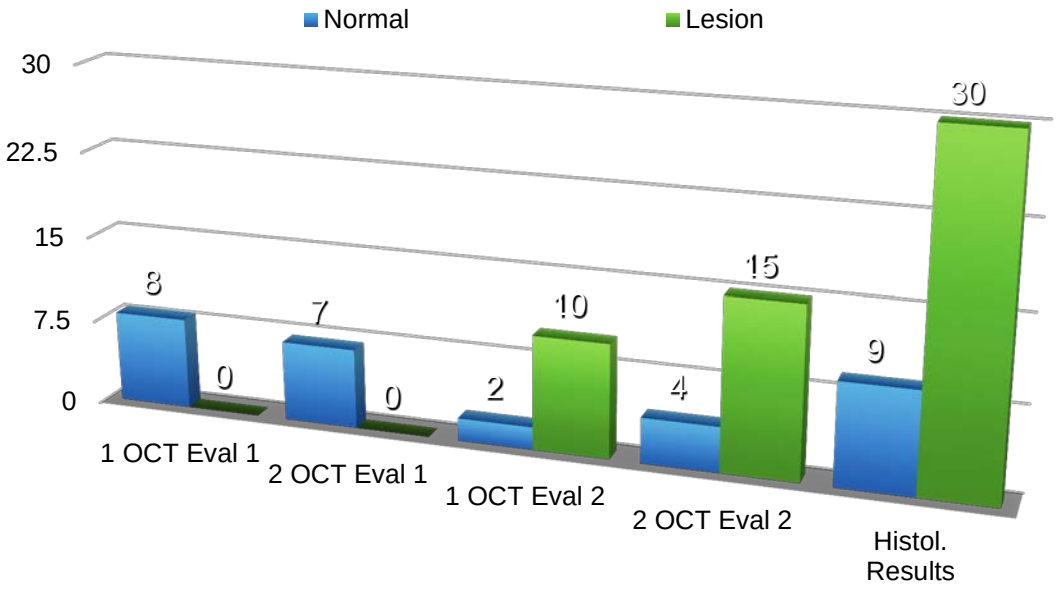


Fig. 18 Accuracy of evaluation of OCT-histological results, treated group

Many of the tongues that were evaluated in the treated group were classified as having possible lesions by both evaluators. The tongues that were included in the following results were those that were identified as having possible lesions with the OCT and histologically diagnosed with lesions. For Evaluator 1, in the first evaluation, 2 tongues were classified as possible lesion while in the second evaluation 1 tongue was identified as having a possible lesion, Evaluator 2 selected 14 specimens in the first evaluation as possible lesion while 9 were selected in the second evaluation (Fig. 19).

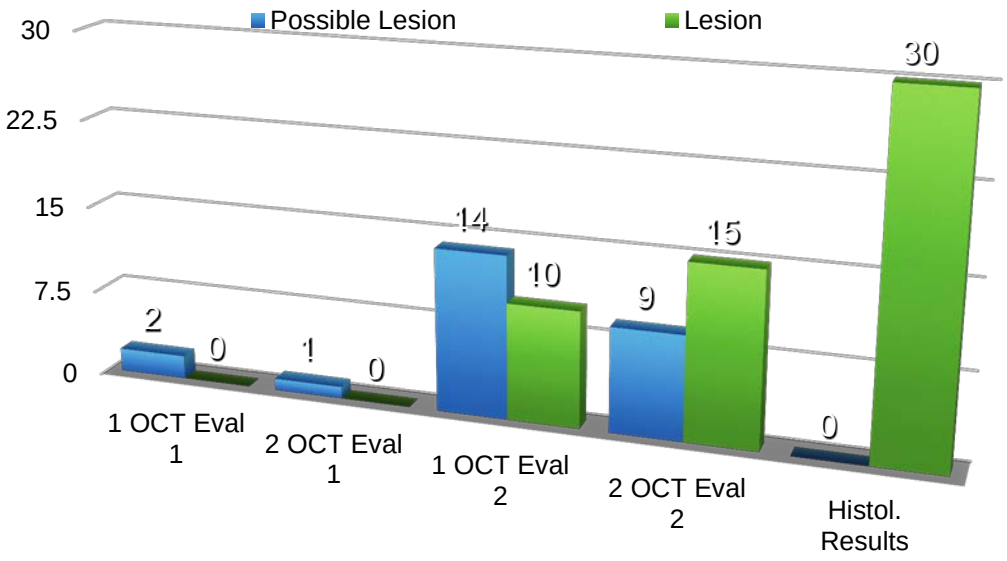


Fig. 19 Evaluation of OCT-Histological results treated group Possible lesion-Lesion

For accuracy of the treated group with histologically diagnosed lesions, evaluator 1 correctly identified 3.33%- 6.66% of the tongues as having possible lesions while evaluator 2 correctly identified 30% and 46.66% of the tongues with lesions and 33.33% and 50% of the specimens were identified as having possible lesions (Fig. 20).

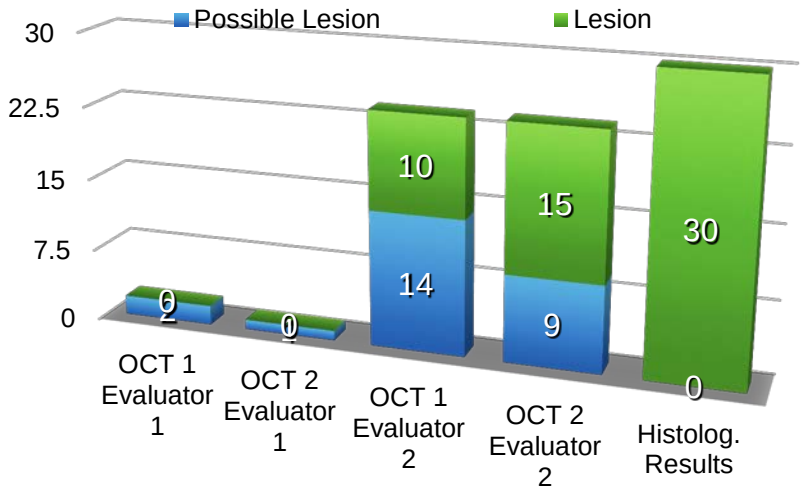


Fig.20 Evaluation OCT - Histological results, Possible Lesion+ Lesion

Comparing the results obtained in the clinical and OCT evaluations, it is evident that the results of evaluator 1 were very consistent: in the normal group, 8 tongues were identified as normal in the first evaluation with the OCT and classified in the same group in the clinical evaluation, while in the second evaluation 7 of the tongues were consistent with the clinical evaluation of the control group. For evaluator 2, however, in the clinical evaluation 6 tongues were included as normal, 3 as having a possible lesion and only one was defined as having a lesion. For this evaluator, only 3 tongues coincided with the clinical evaluation in the first evaluation with OCT, and 4 tongues in the second evaluation (Fig. 21)

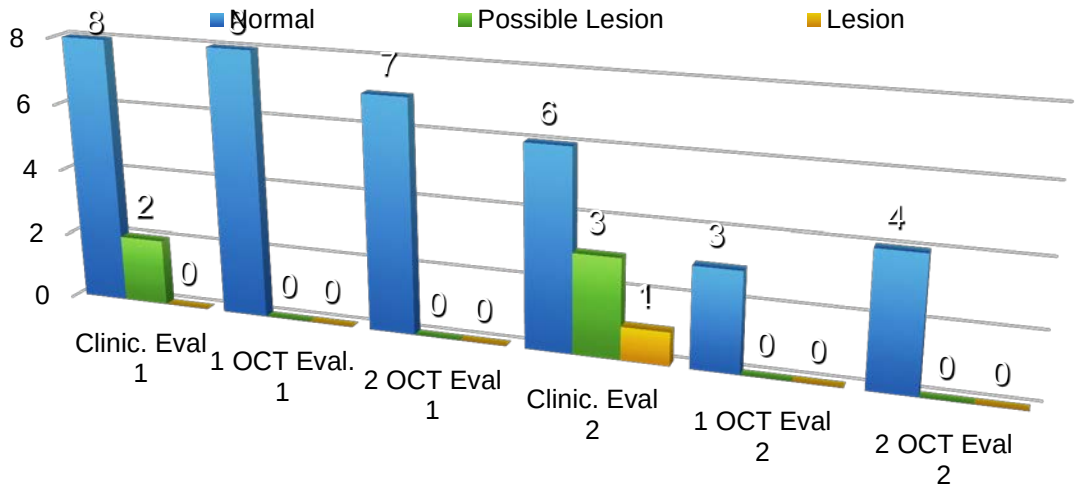
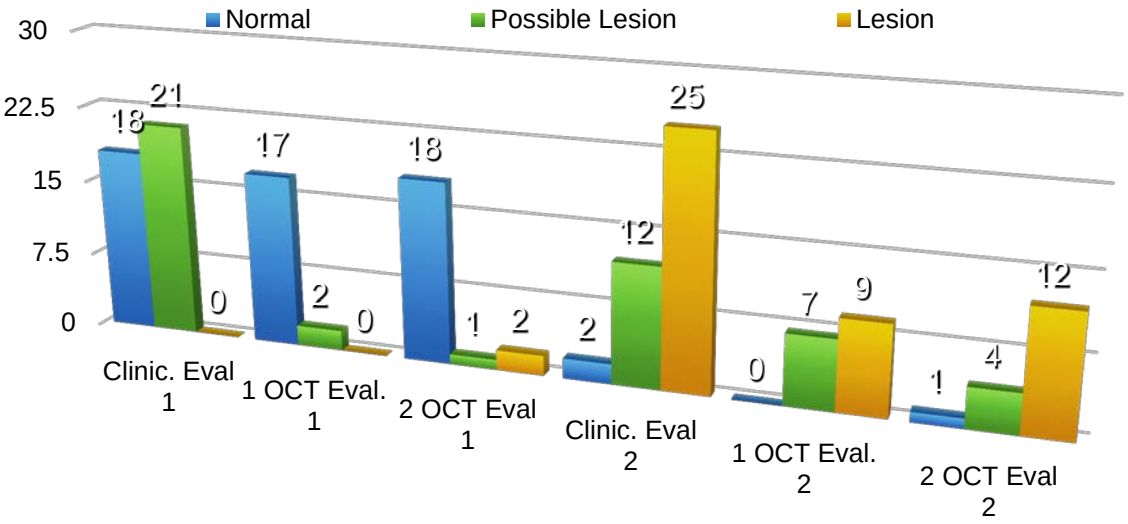


Fig. 21 Comparison of Clinical and OCT Evaluation, Control group

Comparison of the clinical evaluation and OCT images in the treated group differed from the results obtained in the control group. For evaluator 1, 18 tongues were classified as normal in the clinical evaluation while 21 were considered as having a possible lesion. In the first evaluation with OCT, 17 tongues were consistent with the normal diagnosis, and in the second evaluation with OCT, 18 tongues were considered as normal while three tongues were included as having a possible lesion or lesion. In contrast, evaluator 2 considered that only 2 tongues had a normal clinical appearance, 12 had a possible lesion and the remaining 25 had a lesion. In the first evaluation with OCT there was no constancy in the identification of normal tongues in the treated group, while a total of 16 tongues where included as having a possible lesion or lesion. In the second evaluation with OCT, only 1 specimen coincided with the clinical diagnosis as a normal tongue while 4 and 12 tongues were classified as having a

possible lesion or lesion, respectively (Fig. 22). The results of the evaluations are summarized in Table 1.



Fig, 22 Comparison of Clinical - OCT evaluation, treated group

	Categories	Examiner 1			Examiner 2			Histolog Results
		Clinic Eval	OCT Eval 1	OCT Eval 2	Clinic Eval	OCT Eval 1	OCT Eval 2	
Control group	Normal	8	10	9	6	7	8	10
	Possible lesion	2	0	0	3	2	2	0
	Lesion	0	0	1	1	1	0	0
Treated group	Normal	18	36	36	2	8	10	9
	Possible lesion	21	3	1	12	18	12	0
	Lesion	0	0	2	25	13	17	30

Table 1. Results of Clinical and OCT evaluations and histological analysis

STATISTICAL ANALYSIS

Variables evaluated were: 1) presence or absence of the lesion, 2) changes in the thickness of the stratified squamous epithelial layer, 3) changes in the keratinized, stratified squamous epithelium. The presence of the lesion was rated as: No lesion, possible lesion and lesion present. The images of the control group were used as reference of the normal tissue. Images obtained with the OCT resembled the architecture of the tissue evaluated in the histopathologic exam, giving an indication of the structural changes as a consequence of the potentially malignant oral mucosal lesion. With the OCT it is difficult to try to identify precisely which cells are affected, compared to the histopathologic sections that provide a positive identification of the affected cells.

Statistical analysis was performed utilizing the Kappa statistic, which is used when it is necessary to evaluate the results of physical exam findings, radiographic interpretations or other diagnostic tests, where the interpretation or diagnosis depends on the subjective interpretation of the evaluators. A Kappa of 0 indicates the amount of agreement if the evaluators were simply guessing, and a Kappa of 1 indicates a perfect agreement [45]. Based on the standard of this study K=0.6 was used as a good agreement.

According to the Kappa statistics, when the clinical evaluation of Evaluator 1 was compared to the histological results, the result was K=0.0335 that means a slight level of agreement, compared to the result obtained for Evaluator 2 where K=0.4439 with a moderate level of agreement (Table 2).

Measure	Weighted Kappa	Level of agreement
Evaluator 1 Clinic Eval. vs Histolog. results	0.0335	Slight
Evaluator 1 OCT 1 Eval. vs Histolog. results	0.0055	Slight
Evaluator 1 OCT 1 Eval vs Clinical Eval	0.0511	Slight
Evaluator 1 OCT 2 Eval. vs Histolog. results	-0.1125	None/negative
Evaluator 1 OCT 2 Eval vs Clinical Eval	0.0855	Slight
Evaluator 1 consistency	0.3691	Fair
Evaluator 2 Clinic Eval. vs Histolog. results	0.4439	Moderate
Evaluator 2 OCT 1 Eval. vs Histolog. results	0.1874	Slight
Evaluator 2 OCT 1 Eval vs Clinical Eval	0.0982	Slight
Evaluator 2 OCT 2 Eval. vs Histolog. results	0.3905	Fair
Evaluator 2 OCT 2 Eval vs Clinical Eval	0.0983	Slight
Evaluator 2 consistency	0.1062	Slight
Inter-examiner Clinical Eval. consistency	0.0681	Slight
Inter-examiner OCT 1 Eval. consistency	-0.0035	None/negative
Inter-examiner OCT 2 Eval. consistency	0.0179	Slight

Table 2. Results of the Kappa statistics

When the OCT evaluation was compared to the histological results, the results obtained for Evaluator 1 were very different between the first evaluation with OCT, where K=0.055, and for the second evaluation, where K=-0.1125. There was a slight level of agreement for the first evaluation and none or negative agreement for the second evaluation. However, the results obtained by Evaluator 2 demonstrated an increase of the level of agreement from slight to fair agreement. The results obtained were K=0.1874 for the first evaluation with OCT and K= 0.3905 for the second evaluation with OCT.

Results of the comparison between the evaluation of the OCT and the clinical evaluation were: for the first evaluation of Evaluator 1, K=0.0511 while the second evaluation of the OCT had a statistical result of K=0.0855, maintaining a slight level of agreement. The results obtained for the OCT evaluations and the clinical evaluation for Evaluator 2 were very consistent, for the first evaluation K=0.0982 while the second evaluation K=0.0983, with a slight level of agreement in both evaluations.

The determinations of the first evaluator were very consistent with a K=0.3691, while Evaluator 2 had a slight consistency with K=0.1062.

During this project, there was a slight consistency in the clinical evaluation between both examiners with K=0.0681. The consistency between the examiners for the OCT evaluations was quite different, while the K statistic for the first evaluation was negative with a value of -0.0035, the second evaluation was slightly consistent with K=0.0179.

Evaluator 1, in general, had a low rate of correct diagnoses, based on clinical and OCT evaluations, however was fairly self-consistent in the evaluation of the OCT images Evaluator 2 was moderately successful at correctly diagnosing lesions during the clinical evaluation but was not consistent with the evaluation of the OCT images. The level of agreement of both evaluators for their clinical evaluations vs. OCT evaluations was very low; the same results were obtained in the inter-examiner comparison

DISCUSSION

In this study our aim was to demonstrate the efficacy of Optical Coherence Tomography to diagnose potentially malignant oral mucosal lesions of the tongue. The reason for investigating this topic is that the oral squamous cell carcinoma is frequently diagnosed in an advanced stage, and the survival rate may improve if it is diagnosed in an early stage.

Screening based on visual and tactile examinations is recommended, which may result in early detection of oral cancer [12]. The main objective of screening is to reduce mortality and morbidity from the disease by preventing progression of the lesion [46]. One of the recommendations reported in the literature is that dentists must perform an exhaustive clinical evaluation to find possible changes in areas such as the ventro-lateral aspect of the tongue, floor of the mouth and cheeks. These are the most frequent sites for oral squamous cell carcinoma, nevertheless more than 50% of oral cancers had spread to distant places of the body before they were originally diagnosed. This may suggest that many providers, or their patients or both, are either failing to recognize premalignant changes of the oral mucosa or the patients are evading clinical evaluation of these findings [47].

In a survey conducted in 1998 by the National Health Interview Survey (NHIS), it was concluded that only 20.1% of American adults have ever received an oral cancer examination while other groups that include Afro-Americans, Hispanics and patients with low education were significantly less likely to have had such an examination. Another factor to be considered is the knowledge about oral cancer.

In a survey of American adults, 66% to 85% responded that they had heard about oral cancer, nevertheless the majority of them did not know about oral cancer's signs and symptoms, risk factors and oral cancer examination [48].

Despite that some authors recommend the clinical evaluation, the criteria between evaluators may vary making successful early detection more challenging. In this project it was evident that of 39 mice that received the carcinogen agent, one evaluator considered that only 21 tongues showed possible clinical changes while the other evaluator considered that in 15 tongues possible changes were noted, and that 24 tongues had some premalignant changes. Nevertheless, 5 of the 10 tongues of the control group, that didn't receive any carcinogen agent in the drinking water, were evaluated as having a possible lesion or a lesion. Despite that the clinical evaluation is the exam most recommended by many clinicians it may have the potential to generate false positives and false negatives [49]. Although it is important to continue to clarify the public health message and promote primary prevention, an important action that could help in the prevention of the oral cancer is determining the feasibility of a national screening program. Despite this strategy designed to enhance early detection of new cases, some authors consider that diagnosing early malignancy by only its visual appearance is not possible [49].

A clinical evaluation must not be limited to the oral cavity. A physical examination is recommended, which must include evaluation of the head and neck, and exhaustive examination of the oral cavity, inspection and palpation of all mucosal surfaces, skin, scalp, tongue, hard and soft palate, dentition and cervical nodes[18].

Some authors suggest that clinical evaluation has limited value as a method for detecting potentially malignant lesions while others have reported that this examination has a high degree of sensitivity and specificity for detection of oral cancer [50].

In this study there was a marked difference in the results obtained in the clinical evaluation of the specimens and the histological results. One of the evaluators was slightly precise in the identification especially for the specimens in the treated group, while the other examiner was moderately precise in the identification of the control and treated specimens. However, the results demonstrated that the clinical examination may vary between examiners. This kind of difference in the criteria may result in false positives or false negatives.

Rethman stated that one of the limitations found in the clinical evaluation is that premalignant lesions often are asymptomatic and may mimic other conditions, whereas others may not be readily evident in routine examination. Also, because malignant and benign lesions may be clinically indistinguishable, the clinician cannot predict the biological relevance of lesions on the basis of their physical features alone [12]. Another aspect to be considered is that oral cancer at an early stage is often dismissed as a traumatic or infective lesion [46]. One of the largest challenges of the evaluation of oral diseases is the dilemma of attempting to predict which potentially malignant lesions will progress to neoplasia, notably oral squamous cell carcinoma (OSCC). It is difficult to find distinctive clinical features that differentiate benign, precancerous and early cancerous mucosal changes [16, 51].

Many new techniques have been developed to aid the clinician in the screening of areas with possible premalignant lesions. Almost all of these techniques are non-invasive and this makes the patient feel more comfortable and less stressed with the screening. Some of these devices included in oral screening are: VELscope, Identafi, Narrow Band Imaging and OCT.

The VELscope (LED Medical Diagnostics Inc, Barnaby, Canada) is used to excite endogenous fluorophores such as certain amino acids, metabolic products and structural proteins. Nevertheless, some authors argued that not all dysplastic lesions displayed loss of autofluorescence and its use could result in missed lesions and a false positive [36].

Identafi (DentalEZ, PA, USA) is a multispectral screening device that has three different lights designed to be used in a sequential manner to facilitate intraoral examination. With Identafi normal mucosa exhibits natural fluorescence, whereas abnormal tissues appear dark due to diminished autofluorescence. The ability to differentiate between low and high risk lesions is difficult with this device [36].

Narrow Band Imaging (NBI; Olympus Medical Systems Corporation, Tokyo, Japan) is an endoscopic visualization technology which enhances the mucosal surface texture and underlying vasculature. NBI is based on the concept that the wavelength of light determines the depth of penetration, and that changes in the color of the superficial mucosa will be noted according to the extension of the lesions. With a magnification of approximately 1.5 times digital zoom, NBI has the potential to detect malignancies that might be missed with white light [36, 52].

Despite the diversity of screening devices, none of these replaces conventional visual and tactile examination of the oral soft tissues and are not diagnostic tests. None of these devices can effectively differentiate which lesion is considered a low risk or high risk [36, 46]. It is important to recognize that the use of any visual aid is only an adjunct to the rigorous clinical evaluation of the head and neck and will never replace this important exam [51].

Another diagnostic aid that has been used is the transepithelial cytology (Oral Cdx Brush Test, Suffern, NY). It consists of a disposable, circular plastic brush that the dentist or clinician rubs against the suspicious area until pinpoint bleeding is observed, confirming that the basement membrane was penetrated and a transepithelial sample was acquired. While the OralCDx BrushTest has demonstrated validity as an adjunct to lesion assessment in specific clinical situations, practitioners must remember that the diagnostic gold standard for oral cancers and potentially malignant lesions continues to be histopathological examination of surgical biopsy specimens [12, 46].

Optical Coherence Tomography is a recently developed technique that has been used in areas of ophthalmology, dermatology, gynecology and recently in pulmonary, intravascular coronary and esophageal imaging. This high resolution imaging modality generates cross-sectional images with endogenous contrast based on variation in index of refraction. It has similar principles as ultrasound but with higher resolution [53].

Comparison between OCT images and histopathology has been demonstrated in studies of both rats and humans, showing that OCT images could provide

microstructural information of malignant lesions [54]. One of the advantages obtained with the use of the OCT is that this imaging technique reveals many detailed morphologic features of malignant and abnormal tissues, that could be potentially significant local regions with high metabolic activities and early malignant changes [24]. Some of the architectural changes that OCT is capable of showing are: keratinized cell layer, epithelial layer, basement membrane, lamina propria and rete pegs of oral mucosa with the limitation that OCT does not provide any cellular information to grade the potentially malignant lesions [36]. The resolution of the OCT is similar to low power microscopy, approximately 4X [53].

Optical Coherence Tomography (OCT) has the potential to provide immediate and accurate diagnostic information, potentially reducing both the costs associated with unnecessary biopsies and treatment delays [36]. OCT can aid in guiding the clinician in the selection of the site to be biopsied, to ensure the precision of the affected area and to increase diagnostic yield of the biopsied tissue. It is recommended that the clinicians that will be using this imaging technique must be familiar with the normal conditions of the oral cavity in all aspects including clinical, histopathological and OCT imaging. The pathologist plays an important role in the development and implementation of OCT in clinical practice, using this technique as a complement to standard tissue pathology [53]. However, in the present study the results obtained in the OCT evaluation were considerably different between the examiners. One of the evaluators was highly consistent with the classification of the specimens even though the final diagnosis was not consistent with the histological results. On the other hand, the

second examiner was moderately accurate in the classification of the specimens, however was self consistent with the evaluation.

In contrast to the benefits of OCT referred to by many authors, potential disadvantages are the limited backscattering contrast between normal and dysplastic tissues, limited field of view and limitations in imaging depth [36]. Another limitation of OCT is that it requires close proximity to the tissue being imaged to get cross-sectional, depth resolved images with micrometer-scale resolution without destruction or excision of the tissue [55]. Although this technique is known by many authors as an optical biopsy, atypical cells are not distinguished in the OCT [56]. However, the structural changes occurring in the different layers of the epithelium can be visualized in OCT images. Epithelial dysplasia is the most important predictor of malignant transformation and it can only be diagnosed with histological specimens [57].

Regardless of all the advantages obtained with the use of all these new techniques, none of these devices can be considered as a replacement for the histological evaluation. While these devices may aid in the evaluation of the oral cavity, it is important that these devices not be used in the hands of inexperienced clinicians to avoid possible false positives or false negatives [57].

CONCLUSIONS

Oral cancer is one of the most common types of cancer for which survival rates haven't improved in the last years. Dentists and clinicians must consider alternatives to be used to diagnose this disease in an early stage.

The oral cancer screening examination must be part of the daily practice. It is recommended that every practitioner allow additional time to evaluate all the tissues of the oral cavity, head and neck. However, this valuable exam may yield variable results as in this study, in which clinical results differed widely between both examiners. It is important that the clinician must be familiar with the normal aspect of the oral tissues.

Many devices are offered to help the clinician in the screening of oral malignancies. Despite the favorable results that manufacturers report, it is important to emphasize that none of the visual aid devices will substitute for the oral screening; these will be used as an adjunct to the clinical evaluation.

OCT has been demonstrated to be an excellent evaluation aid that may be used to obtain information of the microstructural changes occurring in the oral epithelium; it is helpful to identify suspicious areas where a histological evaluation is recommended. The clinician must be familiar with the technique to avoid any false positives or false negatives. It is recommended to receive training in the use of the OCT, but most important is the necessity of knowing the normal aspect of the tissue visualized via OCT.

Despite the fact that the images obtained with this high resolution imaging technique resemble the architecture of the different layers of the epithelium, it

has an important limitation: there is no information of the cellular changes occurring in the evaluated tissue, and for this reason it may not replace the histological evaluation or biopsy.

However, OCT is a valuable aid in the identification of microstructural changes occurring in the epithelial layers of the oral cavity, with an image quality similar to a low power microscope. This helps the clinician during the oral cancer screening, to obtain immediately multiplanar imaging sections of the evaluated area and 3D reconstruction of the area. Additionally, it may guide the clinician with the correct identification of an affected area to be biopsied.

FUTURE DIRECTIONS

For future studies it is recommended that additional examiners participate in the evaluation of the images obtained in the present study, including specialists in the different areas of dentistry that participate in the screening of patients.

Another aspect to consider in future evaluations is the use of a handheld OCT device to be used *in vivo* in the oral cavity.

A similar study is recommended to evaluate architectural changes of the epithelial layers of the oral cavity from early stage to an advanced stage of oral squamous cell carcinoma.

Additionally, it is recommended that other hard components of the tooth, such as enamel and dentin, be examined to evaluate the accuracy of the OCT in the diagnosis of incipient caries.

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