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ORIGINAL ARTICALE

KI67 EXPRESSION IN RAT NORMAL ORAL EPITHELIUM IN RELATION TO DIFFERENT TIME OF FIXATION IN 10% NEUTRAL BUFFERED FORMALIN

Inas Abdulridha Taha [1], Ban Fadhil AL-Drobie [2]

[1] **University of Baghdad / Collage of dentistry / Oral diagnosis department / Oral and maxillofacial pathology Baghdad, Iraq**

[2] **University of Baghdad / Collage of dentistry / Oral diagnosis department / Oral and maxillofacial pathology Baghdad, Iraq** banaldrobie@codental.uobaghdad.edu.iq

* Correspondence: **Inas Abdulridha Taha** inas.abd2206m@codental.uobaghdad.edu.iq Tel.: +964-7801104742

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ABSTRACT

In many clinical disorders and cancers, immunohistochemistry (IHC) is employed extensively for diagnosis and prognosis. This study was conducted to evaluate the impact of fixation time on Ki67 immunohistochemical expression in rat oral tissues biopsies. 20 albino Rats were employed. The palatal oral mucosa, both soft and hard, was dissected and preserved for one day and seven days in 10% neutral buffered formalin. A Ki67 immunohistochemical staining procedure was used. The findings were statistically analyzed using SPSS after the percentages of positively stained nuclei were graded. Using 10% NBF, there were no significant differences in Ki67 expression between the soft and hard palatal epithelium of rat oral cavity after one day or seven days of fixation. However, there were significant differences between the soft and hard palate specimens after one day and seven days of fixation. As a conclusion , the fixation time within 1 to 7 days has no effect on Ki67 staining of both studied oral sites. On the other hand, Ki67 expression is slightly higher in hard palate epithelium than soft palate regardless of the fixation time.

Keywords: immunohistochemistry; fixation; formalin; Ki67; oral; hard palate; soft palate.

INTRODUCTION

Immunohistochemistry (IHC) is a frequently utilized supporting testing technique for cell categorization and diagnosis in the field of surgical pathology. IHC facilitates the identification of the kind of cell and the organ of origin by using antibodies specific to certain antigens in specific tissue samples and cells. Formalin fixed paraffin embedded (FFPE) tissue is the most commonly utilized tissue type for this procedure, despite the fact that it may also be applied to plastic embedded tissue and was initially developed on frozen sections. This is because FFPE tissue is easier to keep [1-3]. Biological tissue is preserved using a chemical procedure called fixation, which aims to preserve the sample as closely as possible to its in vivo state by stopping the processes of autolysis and putrefaction[4-6]. Formalin is the fixative most commonly used in clinical practice because of its superior ability to preserve tissue characteristics [7].

The most popular fixative for protecting human tissue from autolytic breakdown is 10% neutral buffered formalin, which is a formaldehyde solution with 4% concentration. In the intricate chemical process known as formalin fixation, formaldehyde creates cross-links between proteins and nucleic acids as well as covalent connections [8,4].

The length of time a specimen is submerged in a fixative, or the duration of fixation, has generated a lot of interest in the scientific community due to incompatible results and suggested thresholds. On the other hand, consequences linked to under- or overfixation have clinical impact and include decreases in immunostaining intensity and extent of it [5-13].

A proliferative cell marker, Ki-67 is expressed over the whole cell cycle, with the exception of the G0 stage. Typically, it has been employed to assess a tumor's malignant potential and serve as a prognostic indicator for individuals suffering from malignant neoplasms, such as brain tumors, gastrointestinal and pancreatic neuroendocrine tumors, lymphomas, and breast malignancies [14-19].

According to Dowsett et al. (2011), there is a chance that the preanalytical environment such as fixation process could have an impact on the Ki-67 immunohistochemistry (IHC) and Arima N et al. in 2015 used slides from many institutions to assess the Ki-67 index of breast tumors that were surgically excised. The examination showed that even in tumor cells with a high grade, there were numerous instances with incredibly low or reduced levels of the Ki-67 protein. This gave rise to the theory that the Ki-67 index could be significantly impacted by the postoperative tissue handling of surgically excised breast cancer like fixation conditions. In contrast, Gatta LB et al. (2012) demonstrated that a 24-hour fixation in 10% NBF produced high Ki67 staining results in human lung, colon, liver, kidney, and skin tissues while Joshua D. W et al. (2009) suggested low IHC staining of Ki67 after 7 weeks of fixation in pancreatic and intestine tissues and according to Chunkaruhart C et al. (2021), there shouldn't be any variations in the expression of the Ki67 marker in breast cancer tissue between 10% formalim and 10% NBF[20-24]. Given this, an investigation was conducted into the impact of the duration of the formalin fixation procedure on the staining of Ki67, a proliferation biomarker commonly used to assess the prognosis of a tumor [17,25].

The size of the specimen, the amount of time it takes to fix after a tumor is removed, the type of fixative used, and the length of time of fixation are among the postoperative tissue handling factors of surgical specimens that may affect IHC. The effects of these factors on a number of biomarkers have been studied [26-29].

Rigid analysis hasn't yet been done on the significance of oral tissue handling for the Ki-67 protein. As a result, we carefully investigated the effects of different fixation times on Ki-67 expression in surgically excised normal hard and soft palatal oral mucosa in rats fixed in 10% NBF and predict the optimal time of fixation for accurate results

2. MATERIALS AND METHODS

2.1. Animal Sample

Twenty albino rats, maintained in the Experimental Animal Laboratory at the College of Science, University of Kufa, Annajaf Government, Iraq, were used in the current investigation. The Baghdad University College of Dentistry's ethical committee for animal care authorized the experimental methods and animal handling procedures (Ref. number 867, 3/12/2023)

2.2. Sample Grouping

1-Group A: 10 rats yielded 10 specimens of soft palate and 10 specimen for hard palate fixed for one day in 10% NBF

2-Group B: 10 rats yielded 10 specimens of soft palate and 10 specimen for hard palate fixed for seven days in 10% NBF

2.3. Tissue Preparation

Each group of rats were anesthetized by putting them under a 10% ketamine anesthesia. The palatal oral mucosa, both soft and hard, was meticulously dissected and preserved in 10% (NBF) (available from Diapath, Italy). To create paraffin blocks, palatal tissues were embedded in paraffin wax. Then, 4 µm slices were cut and stained with:

1. Haematoxyline and Eosin (H&E) stain for histological examination.
2. Ki67 biomarker for immunohistochemical analysis (code: ABIN677858)

2.4. Immunohistochemical Method (per the data sheet provided by the manufacturer)

Sections of 4µm in thickness were cut from every block, and the slides were deparaffinized and rehydrated using xylene and graduation alcohol. To retrieve the antigen, (TintoDeparaffinator EDTA 20X, Bio SB) was employed. Endogenous peroxidase activity is inhibited by a peroxidase block. The primary antibody (1:100 for ki67, a rabbit polyclonal antibody from antibodies-online GmbH, Germany) was then treated with the sections. The slides were left at room temperature for thirty minutes in a humid environment. Using a Bio SB detection kit (PI 0265, Rev. H DCN: 3131, Mouse / Rabbit polydetector Plus DAB HRP Detection System), to visibility of antibody binding sites . Hematoxylin counterstain was used and mounted the slides. It was deemed positive when brown staining was seen in the epithelial basal cell layer nucleus.

2.5. Method of Scoring

The number of epithelial cells of basal and supra basal cell layers in five randomly chosen high power fields (40X) that exhibit positive nuclear staining with a specific antibody was used to calculate the immunohistochemical staining for Ki67. All epithelial cells were included in the data representation as a percentage. According to Allawi and Abdullah (2022), the staining percentage was graded as follows: 0 (negative), 1 (less than 10%), 2 (10-35%), 3 (35-70%), and 4 (greater than 70%)[30]. Each slide was assessed in secret, and the readings were calibrated by two experienced pathologists.

2.6. Statistical Analysis

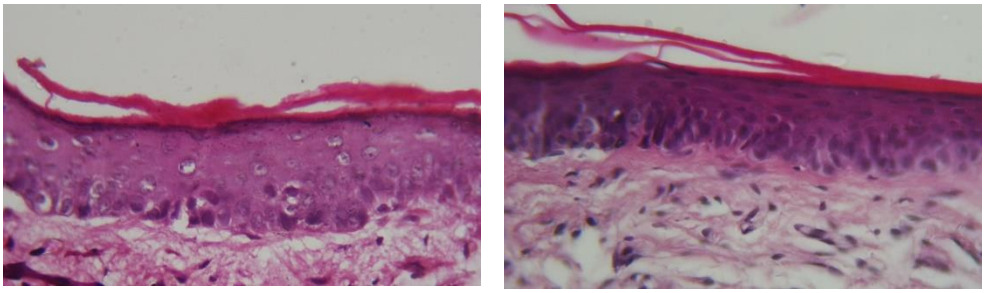
The data were represented by percentages of Ki67 positively stained nuclei. These readings were entered into a computerized database, and IBM SPSS version 28 (IBM Statistical Package for Social Sciences).

The variables were characterized by descriptive statistics: the mean, median, interquartile range, and mean rank. The interquartile range refer to the spread of the middle half of data distribution. Mean rank represent the average of ranks for all observations within each data group. The study data and readings were not normally distributed, so Mann-Whitney U test was used to compare the mean ranks of 1 day and 7 days data. The same statistical test was used to compare the two data groups of oral sites; soft and hard palates. An estimate is considered statistically significant if its P value is smaller than the significance level of 0.001.

3. RESULTS

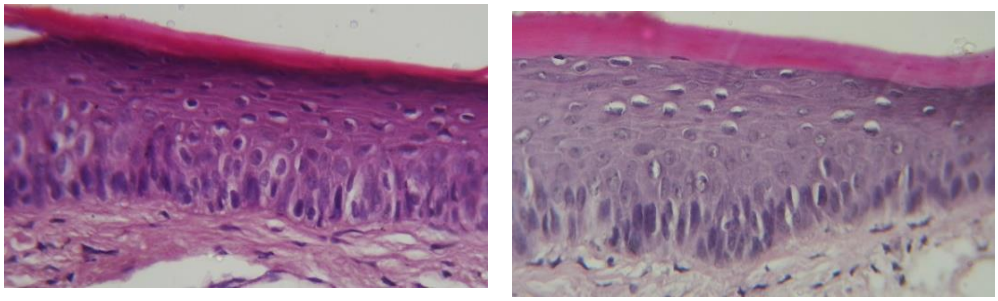
3.1. H&E Results

There were forty slides that were assessed. Twenty slides for each study group (A and B). The oral epithelium seemed normal. It is composed of the lamina propria of connective tissue underneath and surface epithelium. Orthokeratinized stratified squamous epithelium makes up the surface epithelium. The superficial granular cell layer had flat nuclei covered in keratin, the spinous cell layer had rounded nuclei and a polyhedral form. The basal cell layer had oval nuclei with many rete' pegs. Collagen fibers, both fine and thick, are found in the underlying lamina propria as shown in (figures 1 and 2)



a **b**

Figure 1. H&E staining of soft palatal mucosa specimen in (a): group A and (b) : group B (40X)



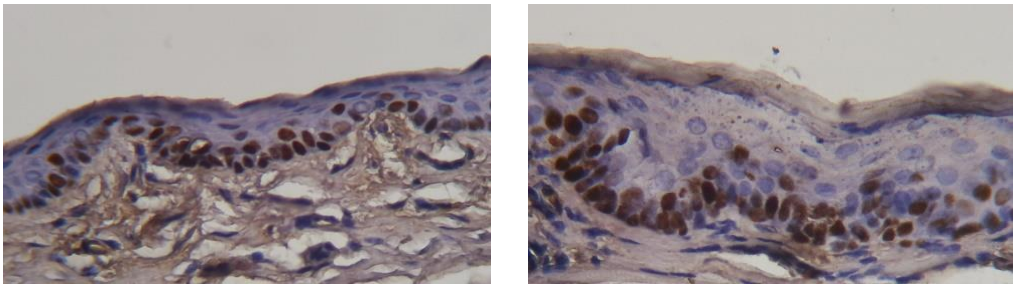
a **b**

Figure 2. H&E staining of hard palatal mucosa specimen in (a) : group A and (b) : group B (40X)

3.2. Immunohistochemical Results of Ki67

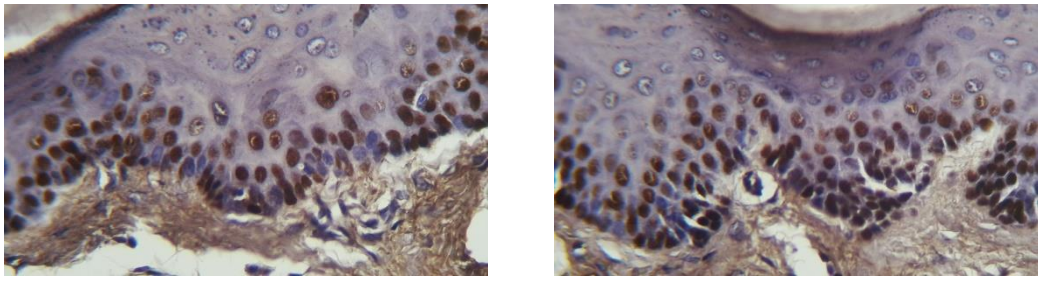
3.2.1. Patterns of Expression

Ki67 immunohistochemical expression was nuclear brown staining. In normal rat oral epithelium of both soft and hard palates in both study groups (A and B). Ki67 expression was clearly observed in basal and suprabasal layers with different percentages of positively stained nuclei while the superficial layers were completely negative as shown in figures (3 and 4)



a **b**

Figure 3. Ki67 expression in epithelium of soft palate specimens in (a) group A and (b) group B.(40X)



a **b**

Figure 4. Ki67 expression in epithelium of soft palate specimens in (a) group A and (b) group B.(40X)

3.2.2. Statistical Analysis

Tables 1 and 2 of the Mann Whitney test showed that there were no statistically significant differences in the immunohistochemical results of the soft and hard palates of albino rats' oral cavities (P values of 0.97 and 0.8, respectively) between the fixation times of 1 day and 7 days by 10% NBF (figure 5).

Table1.Ki67 expression in soft palate rat specimens fixed in 10% NBF for 1 day and 7 days

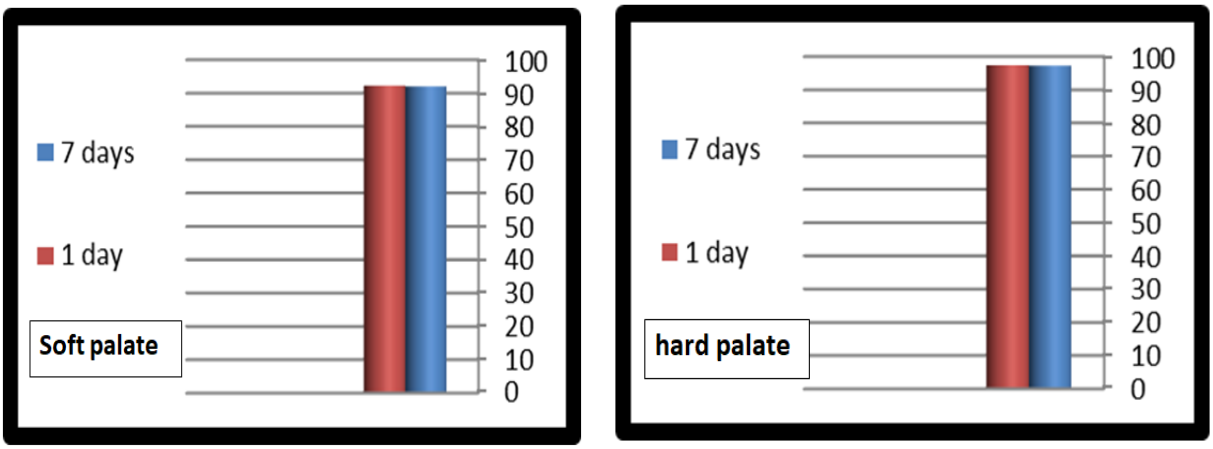
Ki67 expression	Duration of Fixation		P[3](MannWhitney) test
	1 day	7 days	
Soft palate			
N[1]	10	10	
Range	(90.2 - 95.5)	(90 - 95.5)	
Mean	92.2	92.0	**0.97 [NS][2]**
Median	91.8	91.9	
Interquartile range	(90.5 - 93)	(90.5 - 93)	
Mean rank	10.6	10.4	

[1]N:Number of specimens [2]NS: Non-significant, [3]P: P value ($p < 0.001$).

Table 2. Ki67 expression in hard palate rat specimens fixed in 10% NBF for 1 day and 7 days

Ki67 expression	Duration of Fixation		P[3](MannWhitney) test
	1 day	7 days	
Hard palate			
N[1]	10	10	
Range	(95 - 99.3)	(95.2 - 99.5)	
Mean	97.4	97.2	**0.8 [NS][2]**
Median	97.9	97.4	
Interquartile range	(95.5 - 98.6)	(95.5 - 98.3)	
Mean rank	10.9	10.1	

[1]N:Number of specimens, [2]NS: Non-significant, [3]P: P value ($p < 0.001$)



a **b**

Figure 5. (a) : Ki67 expression in group A and B in soft palate, **(b)** : Ki67 expression in group A and B in hard palate

When comparing the Ki67-positive nuclei in the soft palate to the hard palate results, there were significant differences, with p values of <0.001 and <0.001, between the fixation periods of one day and seven days for both sites using the same 10% NBF. The Mann Whitney test was used to express these results, as seen in tables (3 and 4) and figure (6)

Table 3. Ki67 expression in 1 day in relation to soft and hard palate oral specimens fixed in 10% NBF

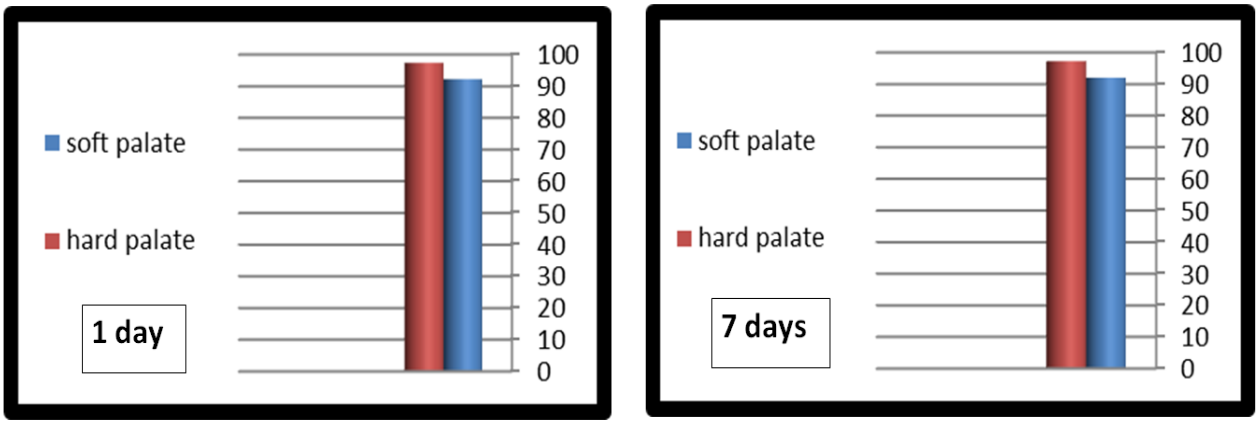
Ki67 expression	**Oral site specimen**		**P[2](MannWhitney) test**
	Soft palate	**Hard palate**	
1 Day			
N[1]	10	10	
Range	(90.2 –95.5)	(95 – 99.3)	
Mean	92.2	97.4	**<0.001**
Median	91.8	97.9	
Interquartile range	(90.5 – 93)	(95.5 – 98.6)	
Mean rank	1.55	4.05	

[1]N:Number of specimens, [2]P: P value (p < 0.001)

Table 4. Ki67 expression in 7 days in relation to soft and hard palate oral specimens fixed in 10% NBF

Ki67 expression	**Oral site specimen**		**P[2](MannWhitney) test**
	Soft palate	**Hard palate**	
7 Day			
N[1]	10	10	
Range	(90 – 95.5)	(95.2 – 99.5)	
Mean	92	97.2	**<0.001**
Median	91.9	97.4	
Interquartile range	(90.5 – 93)	(95.5 – 98.3)	
Mean rank	1.5	4.1	

[1]N:Number of specimens, [2]P: P value (p < 0.001)



(a) **(b)**

Figure 6 (a): Ki67 expression in 1 day fixation in relation to oral sites, **(b)**: Ki67 expression in 7 day fixation in relation to oral sites

The mean rank and interquartile range of the data for the two rat oral sites under study and the two time intervals clearly illustrates these variations. When a soft palate specimen was fixed for one day in 10% neutral buffered formalin, its mean rank was 1.55. For a hard palate specimen, it was 4.05 and after seven days for soft palate, it was 1.5 and for a hard palate specimen, it was 4.1. The interquartile range of Ki67 positively stained cells was as follows: for specimens fixed for one day, the range for soft palate was 90.5 – 93 and for hard palate was 95.5 – 98.6. For specimens fixed for seven days, the range for soft palate was 90.5 – 93 and for hard palate was 95.5 – 98.3. as illustrated in tables (3 and 4)

DISCUSSION

In the new field of personalized medicine for cancer treatment, the measurement of biomarkers by immunohistochemistry (IHC) is frequently applied to tumor specimens to aid in the diagnosis, prognosis, and characterization of many solid tumor types. It is also being used more to monitor and predict drug responses. Fixation is a chemical process used to preserve biological tissue with the goal of keeping the sample as similar to its in vivo form as possible. Maintaining a tissue sample as close to life as possible requires stopping the processes of autolysis, or self-degradation via proteolytic enzymes, as well as putrefaction, or the breakdown of organic materials through the action of microbes. The ideal fixative should preserve the tissue sample in a way that is representative of its in vivo environment, preserve cellular and extracellular structure, and avoid denaturing proteins that are essential for histopathological examination [4-6]. While it is widely acknowledged that the kind of tissue fixative utilized, the length of fixation, and the size of the tissue specimen significantly impact the methods and biomarkers that may be reliably assessed [25,31,32]. The immunohistochemistry is commonly used in many research for diagnosis of different antigens of different normal and pathological tissues [24,33-39]. The impact of fixation time on Ki67 IHC staining in various tissues has not been extensively studied [40].

According to Hitchman E. et al. (2011), staining decreased significantly between 24 and 48 hours after formalin fixation. This suggests that the effects of prolonged formalin fixation had a significant impact on the ability to detect Ki67 expression, which was assessed in human colorectal cancer and leiomyosarcoma specimens. They proposed that specimen type, tissue preservation methods, and ischaemia can all have an impact on the capacity to assess tissue biomarkers [41].

According to research done in 2015 by Arima N et al., breast cancer tissues fixed with 10% (NBF), they demonstrated that over-fixed cancer tissues for 2–14 days fixation led to weak Ki67 nuclear labeling in a time-dependent manner, which disrupted the detection of Ki67 positive cells. Their findings were explained by the fact that prolonged exposure of tissue to formalin inhibits the detection of nucleic acids [21].

These results are at disagreement with those of the current investigation, which demonstrated non-significant effects on Ki67 expression in positively stained cells fixed for one to seven days in 10% neutral buffered formalin in both the soft and hard palatal mucosa of rats. The study design and the study's sample origin—normal animal tissue—may be to blame for this inconsistency.

According to Gatta LB et al. (2012), a 24-hour fixation in 10% NBF produced excellent Ki67 staining results for human lung, colon, liver, kidney, and skin tissues[22]. These findings are consistent with the findings of the current study, which demonstrated excellent staining of the Ki67 of rat oral epithelium in the soft and hard palate after a one-day fixation in 10% NBF.

Low IHC staining of Ki67 was seen in another

investigation conducted in 2009 by Joshua D Webster et al. following the 7-week fixation of pancreas and intestinal tissue samples[23]. Furthermore, after prolonged formalin fixation, antigen retrieval techniques are very effective at maintaining Ki-67 immunoreactivity [9]. These results were consistent with the current study's findings, which demonstrated significant Ki67 staining in tissue samples from both oral locations after seven days of fixation.

The current study examined the normal oral epithelium of rats in both the soft and hard palatal epithelium. It was found that Ki67 expression was restricted to the basal and parabasal cell layers, and that staining tissue samples fixed in 10% neutral buffered formalin for one or seven days had a significant impact. The type of animal tissue used, sample size, antibody type, and standardization of tissue processing procedures all contribute to the explanation of these results.

The present study reports a significant difference in positive staining of Ki67 between the hard and soft palatal epithelium. The hard palatal epithelium appeared slightly higher. This difference may be attributed to epithelial cell turnover, a continuous cell renewal process caused by the site's exposure to the function of mastication [42,43] (figures 3 and 4)

5. CONCLUSIONS

Based on the current investigation, the fixation time within 1 to 7 days has no effect on Ki67 staining of both studied oral sites, soft and hard palates . On the other hand, Ki67 expression is slightly higher in hard palate epithelium than soft palate regardless of the fixation time. The study suggest standardizing formalin fixation periods based on tissue type and immunohistochemical technique for Ki67 staining estimate.

DECLARATIONS

Informed consent

Informed consent was obtained from the patient in this study.

Conflict of interest

The authors declare that they have no conflict of interest.

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