Original Article

The Impact of Fixation Time and Neutral Buffered Formalin Concentration on Pan-CK and P63 Immunostaining in Rat Oral Epithelium

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ABSTRACT

Background: Immunohistochemistry (IHC) needs quality control for detecting a specific antigen. Hence, tissue fixation and processing must be taken into consideration to obtain the optimal immunostaining results.

Objectives: This study aimed to evaluate the interaction and effects of fixation time, fixative concentration, and different rat oral epithelial sites using PanCk and P63 markers on the results of immunohistochemical staining, and to identify the optimum values for these variables.

Methods: A total of 200 specimens from rat normal oral mucosa from different sites were studied. Both concentrations of neutral buffered formalin (NBF) (10% and 5%) were used to fix the specimens, which were assigned to the following groups: (A) 10% NBF for 1 day, (B) 10% NBF for 7 days, (C) 5% NBF for 1 day and (D) 5% NBF for 7 days. Haematoxylin and eosin (H&E) staining and immunohistochemical staining using PanCk and P63 were performed. The variables were measured using a specific scoring system and analysed with SPSS software,version 27.

Results: For both markers and at both concentrations, significant differences were observed between 1 and 7 days of fixation (<0.001). The effect of fixation duration for PanCk and P63 was strong (r statistic >0.5), with higher expression for day 1 in 10% NBF and after 7 days in 5% NBF. For both markers, there were significant differences between 10% and 5% NBF in all study groups (<0.001). The effect of NBF concentration on both markers was strong (r statistic >0.5). The oral sites showed significant differences in P63 expression in all study groups (P<0.001), while PanCk expression did not. The effect of oral site on immunohistochemical expression was strong for P63 and weak for PanCk.

Conclusion: The studied parameters had varying effects on immunohistochemical staining. A concentration of 10% NBF and a fixation time of 1 day yielded the optimal results for both immunohistochemical markers.

Keywords: Fixation, Formalin, Immunohistochemistry (IHC), Oral, Pan-Ck, P63

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Introduction

Pathological diagnosis relies significantly on immunohistochemistry (IHC). Early detection, diagnosis, prognosis, and risk assessment involve the immunohistochemical detection of biomarkers (Otali et al., 2009).

IHC can be used to examine the relationship between a specific antigen and distinct cell types or histological abnormalities, as well as to detect antigen expression in pathological tissue (Mondal, 2017).

Fixation, particularly with an aldehyde fixative (10% neutral buffered formalin [NBF]), the probable interaction of fixation on subsequent biopsy-processing steps, and antigen retrieval all have a significant impact on IHC (Bancroft & Gamble, 2008; Eltoum et al., 2001; Ashour et al., 2019).

The time between tumor removal and fixation, the type of fixative used, and the duration of fixation are postoperative tissue-handling considerations that may affect IHC (Hama et al., 2022; Magakiet al., 2019).

One important element of the epithelial cytoskeleton is cytokeratin (Steinert & Roop, 1988). A combination of two distinct anti-CK monoclonal antibody clones that creates a single reagent is known as CKAE1/AE3 (Kumar et al., 2022; Bhadkariya et al., 2016). Pan-CK (AE1/AE3) is frequently used as a primary marker to distinguish epithelial tumors from non-epithelial tumors (Kumar et al., 2022; Farrar et al., 2016). Pan-CK is expressed in normal epithelium, with cytoplasmic expression that highlights the cell membrane (Menz et al., 2023).

The p63 protein belongs to the p53 tumor suppressor family and has a functional relationship with p53 (Fisher ML et al., 2020; Gatti V et al., 2019). The p63 protein is a transcription factor involved in cell cycle arrest, apoptosis, morphogenesis, epithelial tissue development, and cell differentiation (Yong et al., 2014, Wang et al., 2017). P63 is an immunohistochemical nuclear stain expressed in the basal cell layers of regularly stratified squamous epithelium (Fisher et al., 2020; Di Como et al., 2002; Steurer et al., 2021). Numerous studies have confirmed the clinical significance of p63 as a tumor prognostic and diagnostic indicator (Ramasubramanian et al., 2013; Sundberg et al., 2021; Saghravanian et al., 2017).

No previous studies in oral histopathology have examined the optimization of fixation conditions, such as fixation time and fixative concentration, or their effects on immunohistochemical staining results. However, similar studies have been performed on tissues from sites outside the oral cavity (Chunkaruhart et al., 2021; Khoury et al., 2009; Kashima et al., 2014; Cummings et al., 2020). This study aimed to evaluate the effects and interactions of fixation time, NBF concentration, and tissue site across five rat oral sites on immunohistochemical Pan-CK and p63 staining results, and to identify the optimal fixation time and NBF concentration.

Materials and Methods

Tissue samples

A total of 200 specimens were obtained from the oral cavities of 40 rats at the Experimental Animal Center, Collage of Science, University of Kufa, Al-Najaf Governorate, Iraq. The specimens consisted of normal oral mucosa from five sites: the tongue, buccal mucosa, floor of the mouth, soft palate mucosa, and hard palate mucosa. They were categorized into four groups (A, B, C, and D), each comprising 50 specimens from 10 rats, with 10 specimens from each site. According to the study timetable, all methods, steps, materials, instruments, and equipment used in this study were unified and standardised. The protocol for handling the experimental animals was approved by the Animal Ethics Committee of the College of Dentistry, University of Baghdad.

After sedation of the rats using xylazine (5 mg/kg) and ketamine 10% (50 mg/kg), which were injected intraperitoneally (Kerimoglu et al., 2013), the rats were sacrificed immediately. The oral tissue biopsies were carefully dissected from five oral sites (tongue, buccal mucosa, floor of the mouth, soft palate mucosa, and hard palate mucosa) (Probst et al., 2022).

All biopsies were fixed immediately in 10% and 5% NBF (DIAPATH, Italy) and categorized into four groups (A, B, C, and D):

1- Group A: biopsy fixed in 10% NBF for 1 day

2- Group B: biopsy fixed in 10% NBF for 7 days

3-Group C: biopsy fixed in 5% NBF for 1 day

4- Group D: biopsy fixed in 5% NBF for 7 days

After fixation, paraffin blocks were prepared according to previous reports (Mondal, 2017; Dey, 2018). Histological sections measuring 4 µm were cut, mounted on glass slides, and stained with H&E according to Dey (Dey, 2018) to verify the orientation of the tissue specimens.

IHC

Four-micrometer sections mounted on charged slides were deparaffinized and rehydrated by sequential immersion in xylene and graded alcohol. The slides were washed for 5 minutes three times before each step using Immuno/DNA Washer Buffer Solution (Bio SB: USA)). The immunohistochemical staining protocol was according to the manufacturer's data sheet. The slides were placed in a pre-warmed staining dish containing the retrieving solution (TintoDeparaffinator EDTA 20X, Bio SB) and incubated in a water bath at 90 ˚C for 20 minutes. To inhibit endogenous peroxidase activity, peroxidase block was used for 5 min. The primary antibodies were applied in a humid chamber at room temperature for 30 min: p63 at a dilution of 1:100 and ready-to-use Pan-CK (Anti- Pan-CK (AE1/AE3) (Bio SB, USA), code: PI5433); p63 (Antibodies, Germany), code: ABIN6936179. After applying an anti-mouse/rabbit linker solution to the slides for 15 minutes, the slices were exposed to the fab HRP label solution and incubated for 15 minutes. DAB chromogen solution (Bio SB, USA; PI 0265, Rev. H DCN: 3131, Mouse/Rabbit Polydetector Plus DAB HRP Detection System) was applied for 10 minutes, followed by counterstaining with hematoxylin. The samples were dehydrated using graduated alcohol and xylene, and DPX mounting media and coverslips were carefully applied to the sections. Normal prostate tissue for PanCK and prostate cancer tissue for p63 were used as positive tissue controls (Figure 1). The primary antibody was omitted from the negative tissue control to rule out nonspecific binding.

Scoring system

The slides were examined under a microscope, and expression levels were assessed semi-quantitatively based on the percentage of positively stained cells (Saeed & Abdullah, 2022; Allawi & Abdullah, 2022).

Immunohistochemical staining for both antibodies was assessed in the four groups (A, B, C, and D) by counting the epithelial cells showing positive staining in five randomly selected high-power fields (40X). The data were represented as the percentage of all positive epithelial cells. Staining percentage was scored as follows: score = 0, negative staining; score = 1, < (10%) + ve cells; score = 2, (10–35%) + ve cells; score = 3, (35–70%) + ve cells; and score = 4, > (70%) + ve cells.

Statistical analysis

The data were not normally distributed and were represented as percentages of positively stained cells. These data were entered into a computerized database and analyzed using SPSS software version 27.

The Mann-Whitney test was used to determine the statistical significance of differences in the median between two groups. This test was used for the study variables of fixation time and fixative concentration. The effect size of these differences was measured using the r statistic, where $r<0.3$ indicated a weak effect, r=0.3-0.5 indicated a moderate effect, and $r>0.5$ indicated a strong effect.

The Friedman test was used to assess the statistical significance of differences among the 5 oral tissue sites. The effect size for these differences was measured using Kendall's W, where $W<0.3$ indicated a weak effect, W=0.3–0.5 indicated a moderate effect, and $W>0.5$ indicated a strong effect.

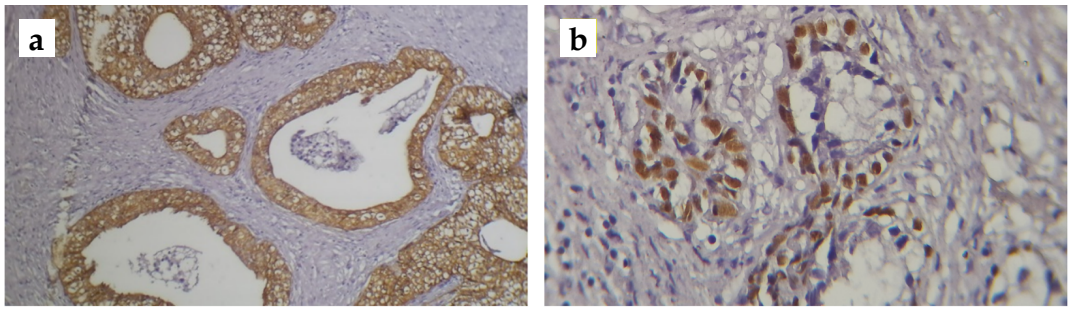


Figure 1. Positive immunohistochemical staining with the primary antibodies in the positive tissue controls used in the study: a) Normal prostate tissue stained with Pan-CK, b) Prostate cancer tissue stained with P63

The assumed levels of statistical significance were as follows: P>0.05 (non-significant), P<0.05 (significant), and P<0.001 (highly significant).

Results

Statistical differences and effect of fixation time

Across all five oral sites, specimens fixed in 10% NBF showed highly significant differences (Mann-Whitney U test, P<0.001) and strong effects (r statistic >0.5) in the median percentages of Pan-CK - and p63-positive specimens between the 1-day and 7-day fixation times (study groups A and B). Median expression was clearly after 1 day of fixation (Table 1 and Figures 2, 3, 4, and 5).

For specimens from all five oral sites fixed in 5% NBF (study groups C and D), the median percentages of Pan-CK - and p63-positive specimens also differed significantly between the 1-day and 7-day fixation times (Mann–Whitney U test, P<0.001), with a strong effect (r statistic >0.5). Median expression was clearly higher after 7 days of fixation (Table 1 and Figures 2, 3, 4, and 5)

Statistical differences and effect of fixative concentration

Regarding the different NBF concentrations, specimens from all five oral sites fixed for 1 day (study groups A and C) showed highly significant differences (Mann–Whitney U test, P<0.001) and strong effects (r statistic >0.5) in the median percentages of Pan-CK - and p63-positive specimens between the 10% and 5% fixative concentrations. The median percentages were higher with a fixative concentration of 10% (Table 2 and Figures 6, 7, 8, and 9.

For specimens from all five oral sites fixed for 7 days in 5% and 10% NBF (study groups B and D), the median percentages of Pan-CK -positive specimens differed significantly (Mann–Whitney U test, P<0.001), with a strong effect (r statistic>0.5). The same results were observed for P63, except in the floor of mouth and soft palate mucosa, which showed nonsignificant differences (Mann Whitney U test, P=0.06 and P=0.12) and moderate effects (r statistic =0.4 and 0.3, respectively). However, the median expression for both markers was consistently higher in 10% NBF after 7 days (Table 2 and Figures 6, 7, 8, and 9).

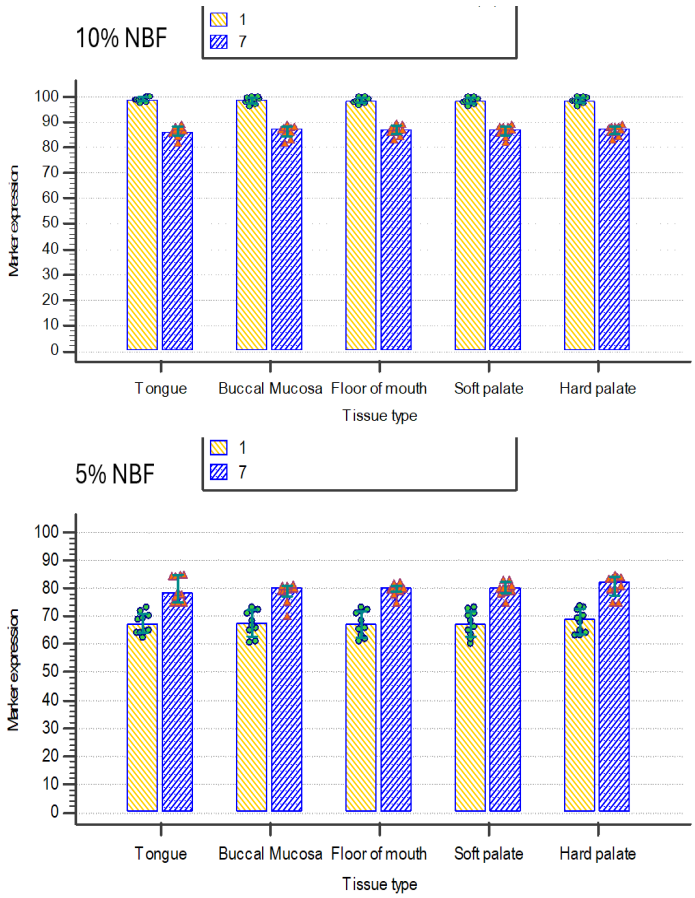


Figure 2. Median Pan-CK expression after 1 day and 7 days of fixation in 10% NBF and 5% NBF across the 5 oral sites

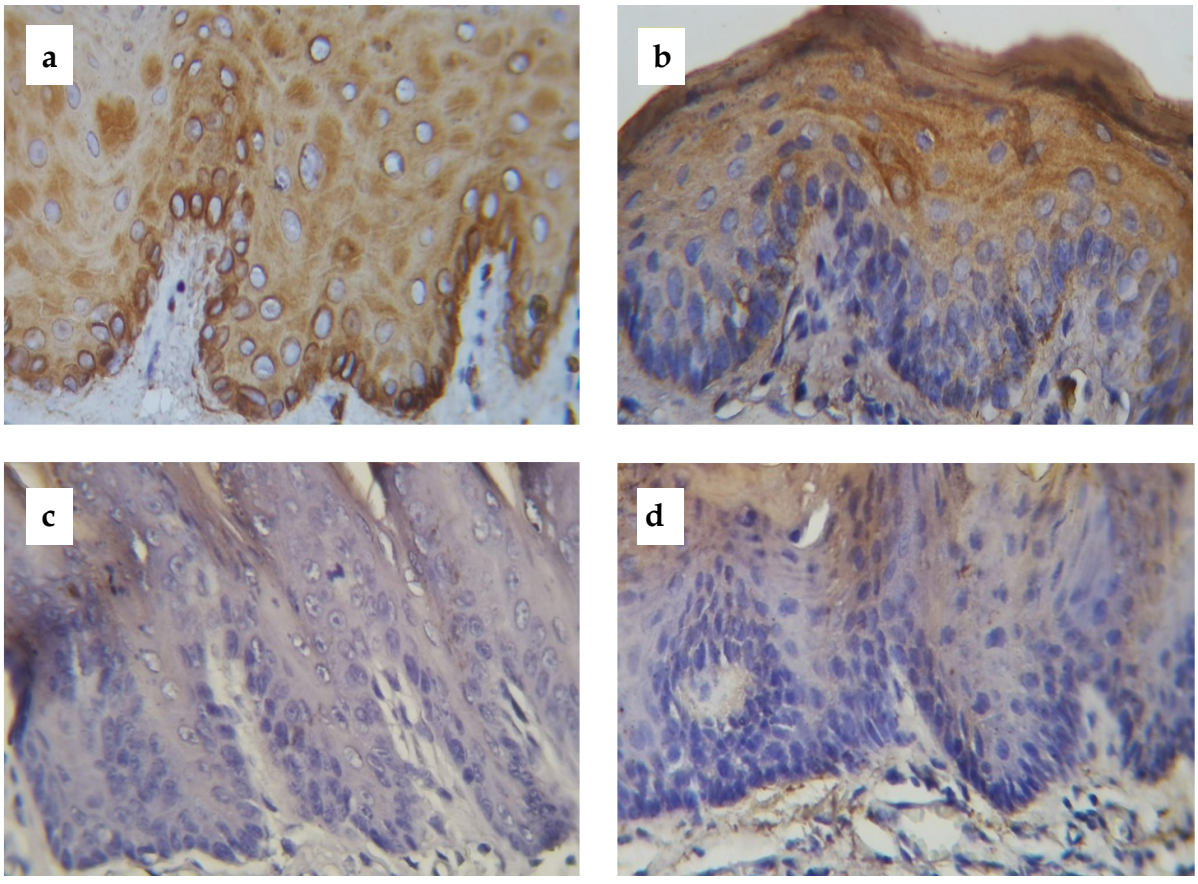


Figure 3. Immunohistochmical expression of Pan-CK in normal rat oral epithelium (×40)

a) Buccal mucosa fixed in 10% NBF for 1 day, b) Buccal mucosa fixed in 10% NBF for 7 days, c) Tongue fixed in 5%NBF for 1 day, d) Tongue fixed in 5%NBF for 7 days

Statistical differences and effects on oral sites

For Pan-Ck, no significant differences in median expression were found among specimens from the five oral sites (tongue, buccal mucosa, floor of mouth, soft palate mucosa, and hard palate mucosa) within any of the study groups (A- D) (Friedman test P=0.88, P=0.89, P=1, and P=0.89, respectively). The effects were weak (Kendall's W = 0.029, 0.028, 0.004, and 0.028, respectively).

In contrast, for P63, significant differences were found among specimens from the five oral sites (tongue, buccal mucosa, floor of mouth, soft palate mucosa, and hard palate mucosa) within each study group (A- D) (Friedman test P<0.001, P<0.001, P<0.001, and 0.014, respectively). The effects were strong in groups A, B, and C (Kendall's W>0.5) and moderate in group D (Kendall's W= 0.311). Median expression levels in the tongue, buccal mucosa, and hard palate mucosa were higher than those in the floor of the mouth and soft palate mucosa (Table 3 and Figure 10).

Discussion

IHC is an essential tool in diagnostic pathology and is relied on heavily for the diagnosis, classification, and prognosis of many tumors, as well as for their assessment (Magaki et al., 2019; Araújo et al., 2021; Guerrero et al., 2023).

Many researchers have used IHC to detect a large number of antigens in different healthy and pathological tissues (Allawi & Abdullah, 2022; Al-Qazzaz et al., 2023; Alshami et al., 2022; Abdulla et al., 2016; Rakha et al., 2017).

Not only the choice of fixative but also its concentration and the duration of fixation are important factors. Fixation may interact with subsequent tissue-processing steps, which can have a significant impact on the results of immunohistochemical analysis (Kyclová et al., 2004; Kashima et al., 2014; Cummings et al., 2020; Grizzle et al., 2001; Grizzle et al., 2008).

Table 1. Differences and effects of fixation time (1 day and 7 days) on Pan-CK and P63 staining using 10% and 5% NBF for all five oral sites

NBF Concentration	Oral Site	Pan-CK Expression				P63 Expression			
		1 Day Median	7 Days Median	P (Mann-Whitney Test)	Effect Size (r Statistic)	1 Day Median	7 Days Median	P (Mann-Whitney Test)	Effect Size (r Statistic)
10% NBF	Tongue	98.9	86.3	<0.001	0.848	97.3	85.8	<0.001	0.846
	Buccal mucosa	98.7	87.6	<0.001	0.847	96.5	86.6	<0.001	0.845
	Floor of mouth	98.3	86.8	<0.001	0.846	92.2	83	<0.001	0.845
	Soft palate	98.5	87	<0.001	0.846	91.2	80.9	<0.001	0.847
	Hard palate	98.1	87.5	<0.001	0.849	96.2	85.8	<0.001	0.845
5% NBF	Tongue	66.9	78.3	<0.001	0.846	70.4	83.3	<0.001	0.846
	Buccal mucosa	67.6	80	<0.001	0.778	70.8	81.4	<0.001	0.846
	Floor of mouth	67.1	80	<0.001	0.845	65.1	80.2	<0.001	0.845
	Soft palate	67.2	80	<0.001	0.845	64.9	80.2	<0.001	0.845
	Hard palate	68.7	82.2	<0.001	0.845	70.3	82.3	<0.001	0.845

Table 2. Differences and effects of fixative concentration (10% NBF and 5% NBF) on Pan-Ck and P63 after 1 day and 7 days of fixation at all five oral sites

Fixation Time	Oral Site	Pan-Ck Expression				P63 Expression			
		10% NBF Median	5% NBF Median	P (Mann Whitney Test)	Effect Size (r Statistic)	10% NBF Median	5% NBF Median	P (Mann Whitney Test)	Effect Size (r Statistic)
1 day	Tongue	98.9	66.9	<0.001	0.848	97.3	70.4	<0.001	0.845
	Buccal mucosa	98.7	67.6	<0.001	0.846	96.5	70.8	<0.001	0.845
	Floor of mouth	98.3	67.1	<0.001	0.846	92.2	65.1	<0.001	0.845
	Soft palate	98.5	67.2	<0.001	0.846	91.8	64.9	<0.001	0.846
	Hard palate	98.1	68.7	<0.001	0.849	96.2	70.3	<0.001	0.846
7 days	Tongue	86.3	78.3	<0.001	0.711	85.8	83.3	0.003	0.635
	Buccal mucosa	87.6	80	<0.001	0.847	86.6	81.4	0.004	0.618
	Floor of mouth	86.8	80	<0.001	0.845	83	80.2	0.06 (NS)	0.431
	Soft palate	87	80	<0.001	0.812	80.9	80.2	0.12 (NS)	0.356
	Hard palate	87.5	82.2	<0.001	0.745	85.8	82.3	<0.001	0.702

Ns: Non significant.

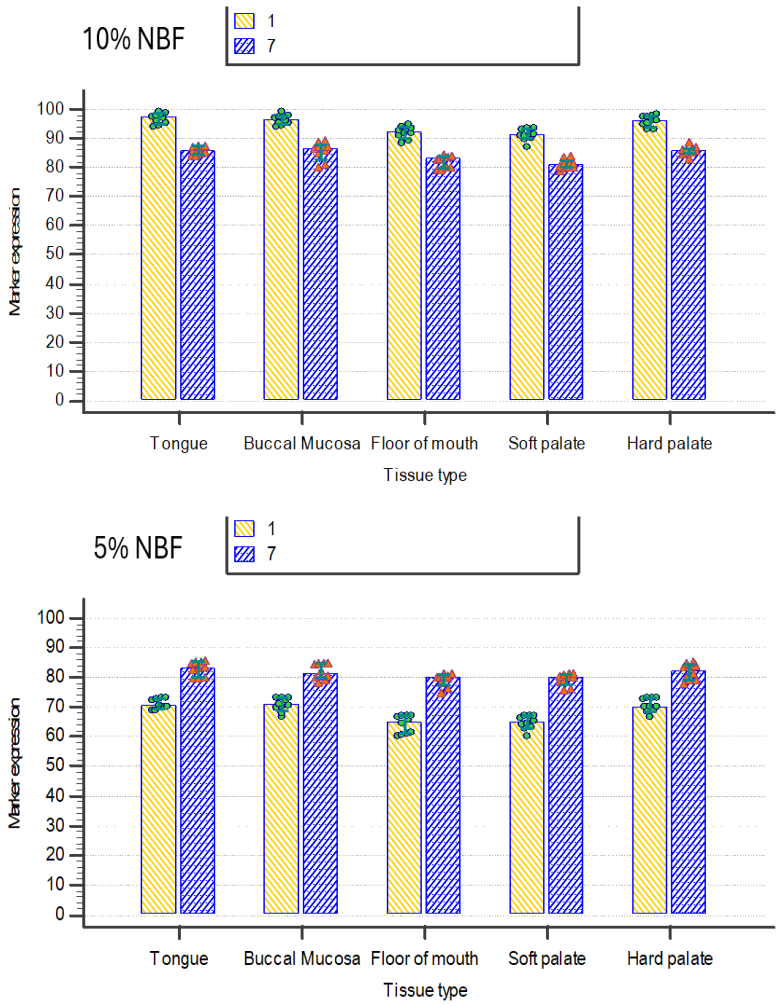


Figure 4. Median p63 expression after 1 day and 7 days of fixation in 10% NBF and 5% NBF across the 5 oral sites

Researchers have extensively studied both factors—the duration of fixation and the fixative concentration—in different tissue types, including the breast, tonsil, kidney, liver, colon, placenta, thyroid, prostate, uterus, brain, and stomach (Taha et al., 2024; Samuel & Sukumaran, 2016).

The rationale for studies investigating fixation duration and concentration may be attributed to the undesirable effects of decreasing or increasing either of these variables.

To our knowledge, the current study is the first to assess normal oral tissue using tissue from experimental animals.

The present study aimed to evaluate the effects of both fixation duration and NBF concentration on the immunohistochemical staining of rat oral epithelium specimens (tongue, buccal mucosa, floor of mouth, soft palate, and hard palate) using the immunohistochemical markers Pan-CK and P63, The goal was to estimate the effects of the study factors and identify the optimal combination for obtaining the best immunohistochemical results.

The study asked to what extent differences in the study factors may influence the degree of positive immunohistochemical staining of cytoplasmic and nuclear targets in oral specimens, and whether these factors have a weak, moderate, or strong effect.

Using the Pan-Ck and P63 markers with both 5% NBF and 10% NBF, the present study found a significant difference in immunohistochemical expression between 1 day and 7 days of fixation across all 5 oral biopsy sites.

These results agree are consistent with those of several studies (Kashima et al., 2014; Cummings et al., 2020; Kyclová et al., 2004; Neal et al., 2003; Arber 2002; Wasielewski et al., 1998; Paavilainen et al., 2010; Gatta et al., 2012; Boenisch 2005; Lenz et al., 2022; Joshua et al., 2009, Ramos-Vara et al., 2000B) and disagree with

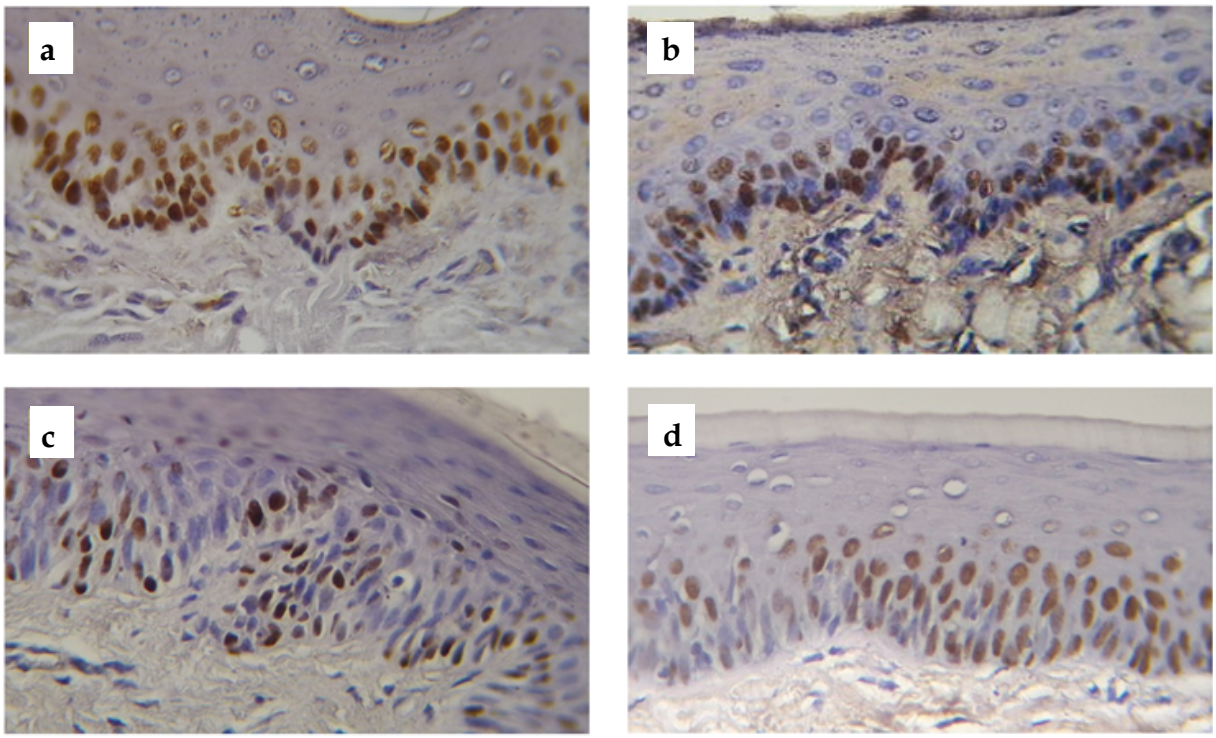


Figure 5. Immunohistochmical expression of P63 in normal rat oral epithelium (×40)

a) Floor of mouth fixed in 10% NBF for 1 day, b) Floor of mouth fixed in 10% NBF for 7 days, c) Hard palate fixed in 5% NBF for 1 day, d) Hard palate fixed in 5% NBF for 7 days

those of other studies (Tong et al., 2011; Julio & Rogers, 2010; Arima et al., 2015; Hitchman et al., 2011).

In the present study, immunohistochemical results differed significantly between 1 day and 7 days of fixation in 10% NBF across specimens from all oral sites, with higher results after 1 day. This may be explained by the slight overfixation that occurred after 7 days compared with 1 day, which led to decreased immunohistochemical staining positivity. This finding indicates that overfixation leads to excessive cross-linking and epitope masking, and that the high concentration of NBF (10%) requires less time for fixation to produce better results. In contrast, with 5% NBF, the immunohistochemical results were higher after 7 days than after 1 day. This may have been caused by underfixation and insufficient fixation during the 1-day period due to the lower concentration.

The strong effect of fixation time demonstrated by the results obtained using Pan-CK and p63 underscores the need to identify the optimal fixation time for each NBF concentration to produce the best immunohistochemical results. For this reason, the current study proposed an evidence-based, suitable, and standardized fixation time to be considered alongside many other factors to obtain optimal results.

For fixation durations of 1 day and 7 days, the current study revealed significant differences in immunohistochemical readings between 10% and 5% NBF for all five oral-site specimens using both studied markers (Pan-CK and p63).

An important exception to the above description is that P63 expression in two oral sites (the floor of the mouth and soft palate) showed no significant differences between 5% and 10% NBF after 7 days of fixation, although median expression was higher with 10% NBF.

Overall, the findings of this study regarding differences were consistent with those of the following studies (Kashima et al., 2014; Cummings et al., 2020; Kyclová et al., 2004; Wasielewski et al., 1998; Gatta et al., 2012; Burns et al., 2008; Matsuda et al., 2011).

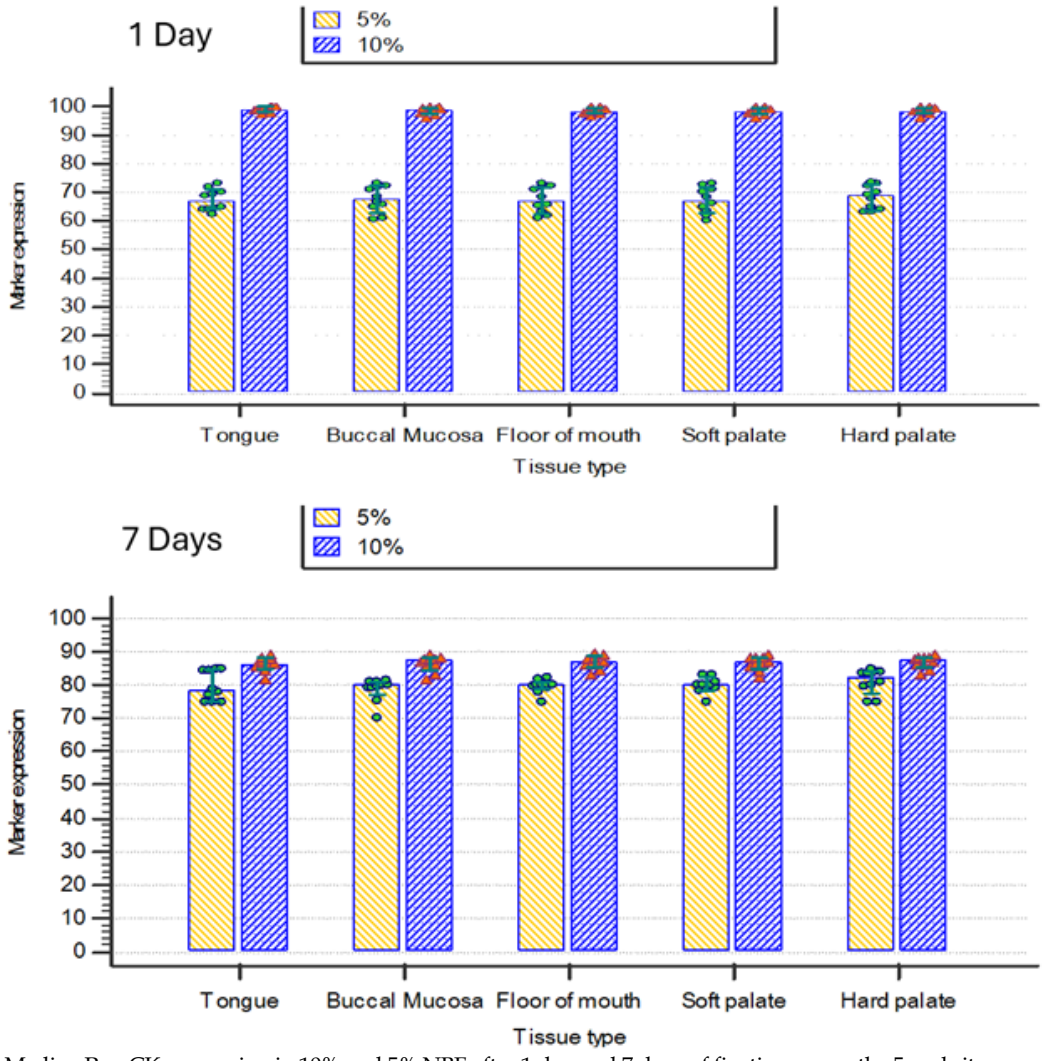


Figure 6. Median Pan-CK expression in 10% and 5% NBF after 1 day and 7 days of fixation across the 5 oral sites

The readings associated with 10% NBF were higher than those associated with 5% NBF for both fixation periods. This finding could indicate that 10% NBF is a more suitable concentration than 5% NBF for fixing oral tissue samples.

Many previous studies support the current results, confirming that 10% NBF provides the best immunohistochemical expression (Kashima et al., 2014; Wasielewski et al., 1998; Kyclová et al., 2004; Gatta et al., 2012). However, these studies used different tissue types and immunohistochemical markers.

The strong effect of changing the fixative concentrations from 10% to 5% NBF revealed the important role of fixative concentration. From the perspective of the present study, a standardized and suitable NBF concentration is proposed for consideration alongside other factors to achieve optimal results.

More specifically, the studies mentioned above differed from the present study in their research parameters, including fixation time intervals, fixative concentrations, tissue types (human or animal samples, but not oral tissue), and the immunohistochemical markers used.

Both study markers (Pan-CK and P63) showed different results across the tested oral sites (tongue, buccal mucosa, floor of the mouth, soft palate mucosa, and hard palate mucosa). When the fixative concentration and fixation time were controlled within each of the four study groups (A, B, C, and D), differences were statistically significant for p63 but not for Pan-CK.

One possible explanation for the significant differences is p63 expression is attributed to epithelial cell turnover, a continuous cell renewal process in the tongue, buccal mucosa, and hard palate mucosa. These sites are frequently exposed to the forces of mastication, and this ex-

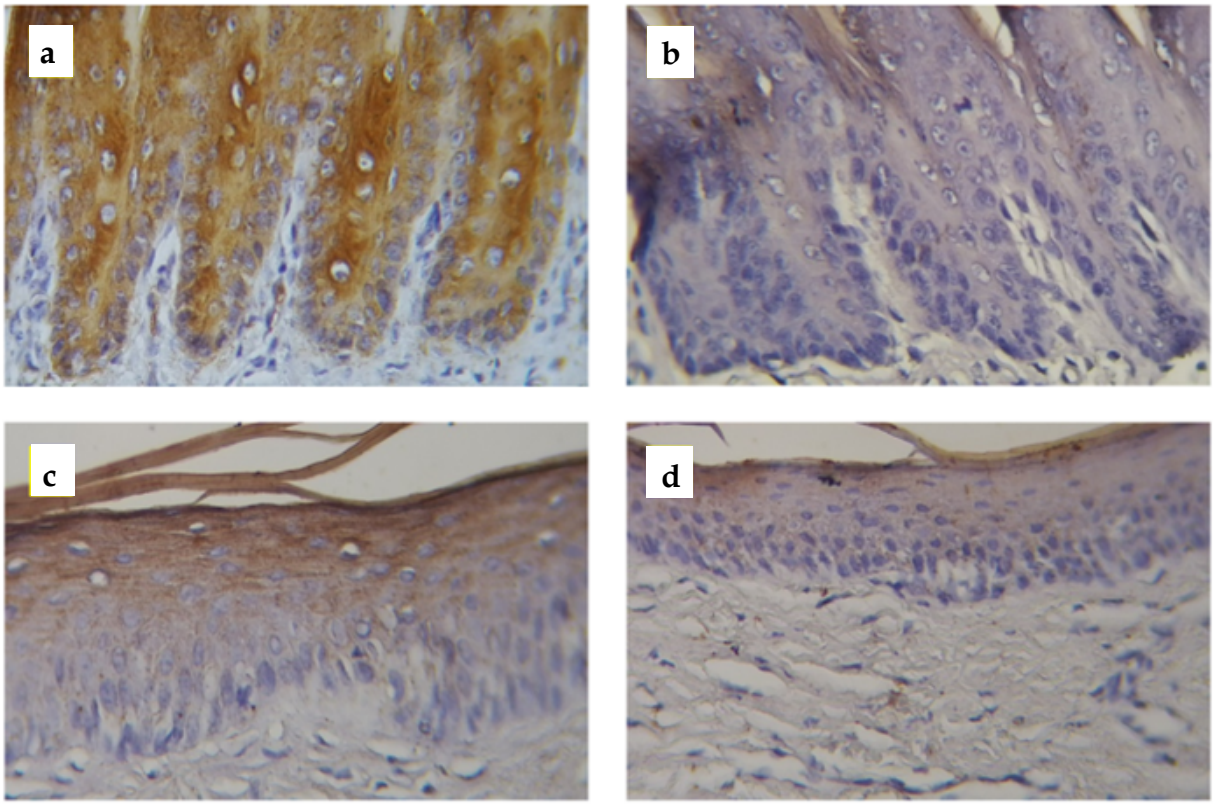


Figure 7. Immunohistochmical expression of Pan-CK in normal rat oral epithelium (×40)

a) Tongue fixed in 10% NBF for 1 day, b) Tongue fixed in 5% NBF for 1 day, c) Soft palate fixed in 10% NBF for 7 days, d) Soft palate fixed in 5% NBF for 7 days

planation is supported by a substantial body of research (Orbans, 2011; Izumi et al., 2002).

p63 is believed to be produced in proliferative cells in the basal layer of normal oral epithelium, where it helps maintain their basal cell state (Fisher et al., 2020; Ramasubramanian et al., 2013). p63 is crucial for the morphogenesis, differentiation, and development of epithelial tissue (Yong et al., 2014).

On the other hand, the immunohistochemical expression of Pan-CK showed no significant differences among the studied oral sites (tongue, buccal mucosa, floor of the mouth, soft palate mucosa, and hard palate mucosa).

Cytokeratins are an important component of the epithelial cytoskeleton and are considered cytoskeletal proteins (Steinert & Roop, 1988). Pan-CK is a specific epithelial immunohistochemical marker. In the epithelium, AE1/AE3 normally shows cytoplasmic expression, with emphasis on the cell membrane; its expression is not restricted to the basal and parabasal layers or to cells with proliferative status (Kumar et al., 2022; Farrar et al., 2016).

The effect of oral tissue site on immunohistochemical staining varied by intraoral location and marker type, even when fixation time and concentration were held constant within each study group.

Therefore, oral sample sites should be considered when interpreting immunohistochemical examinations, especially when different markers are used. This finding may be attributed to the nature and origin of the tissues, their functional activity, and their distinctive molecular features.

In conclusion, the study parameters had important effects on the immunohistochemical process. These findings underscore the need for manufacturers to develop ideal methodological protocols based on targeted scientific research to obtain accurate and reliable results that can inform subsequent steps in the diagnostic process and influence treatment planning.

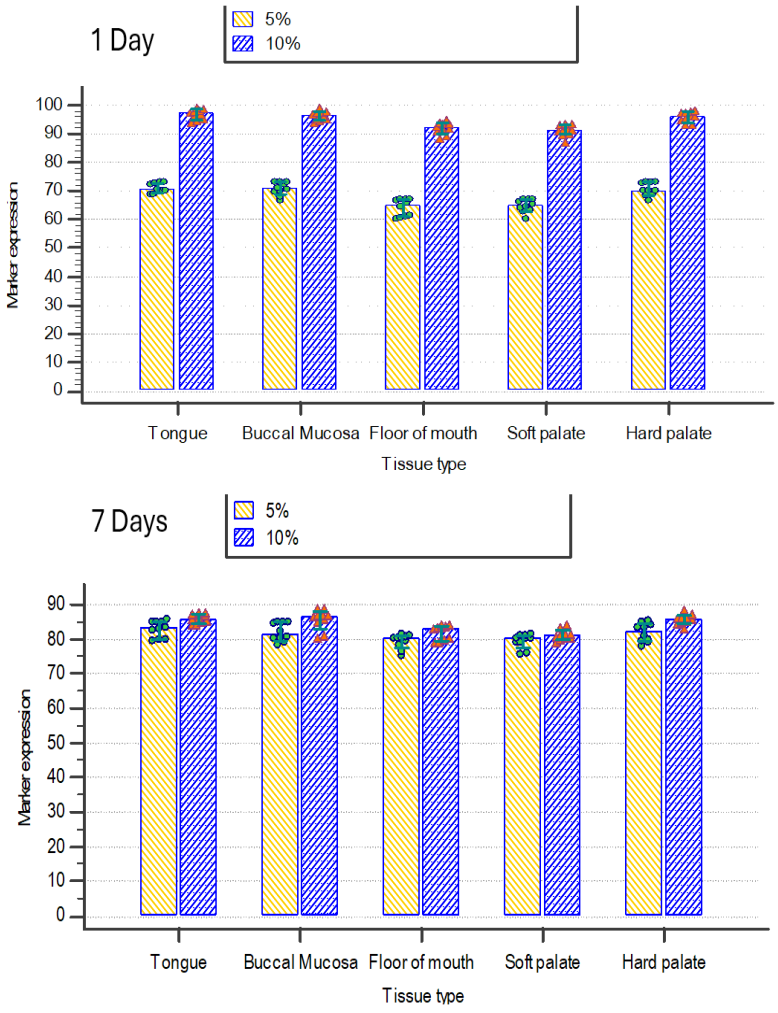


Figure 8. Median p63 expression in 10% and 5% NBF after 1 day and 7 days of fixation across the 5 oral sites

Table 3. Differences and effects of five oral sites for Pan-Ck and P63 in all study groups (A- D)

Group		Oral Sites (Median)					P (Friedman test)	Effect Size (Kendall's W)
		T	BM	FOM	SP	HP		
Pan-Ck expression	A	98.9	98.7	98.3	98.5	98.1	0.88	0.029
	B	86.3	87.6	86.8	87	87.5	0.89	0.028
	C	66.9	67.6	67.1	67.2	68.7	1	0.004
	D	78.3	80	80	80	82.2	0.89	0.028
P63 expression	A	97.3	96.5	92.2	91.2	96.2	<0.001	0.706
	B	85.8	86.6	83	80.9	85.8	<0.001	0.662
	C	70.4	70.8	65.1	64.9	70.3	<0.001	0.762
	D	83.3	81.4	80.2	80.2	82.3	<0.014	0.311

Abbreviations: T: Tongue; BM: Buccal mucosa; FOM: Floor of mouth; SP: Soft palate; HP: Hard palate.

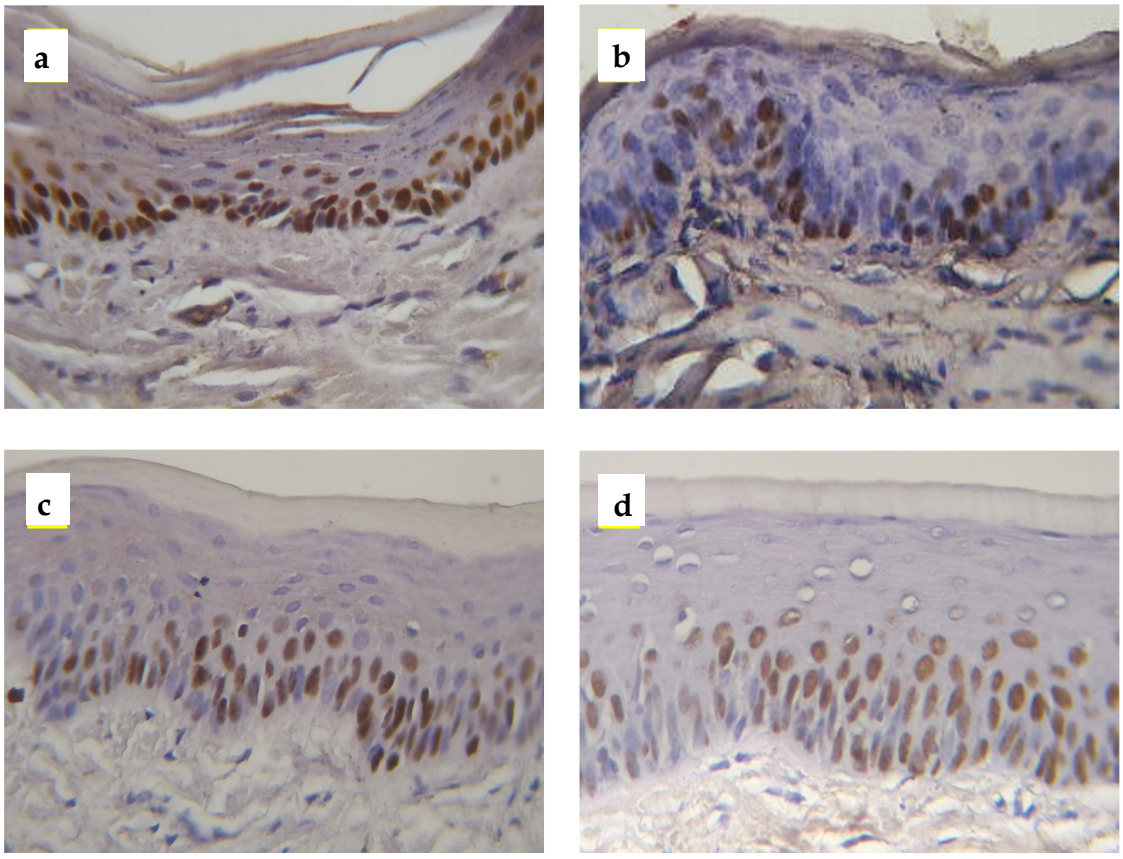


Figure 9. Immunohistochmical expression of P63 in normal rat oral epithelium (×40)

a) Soft palate fixed in 10% NBF for 1 day, b) Soft palate fixed in 5% NBF for 1 day, c) Hard palate fixed in 10% NBF for 7 days, d) Hard palate fixed in 5% NBF for 7 days

The limitations of the present research were as follows

1. Only two immunohistochemical markers were used because including more markers would have increased the budget.

2. Studying additional NBF concentrations and fixation time intervals might have been beneficial, but would have increased the cost.

Conclusion

The current study revealed significant effects and interactions among the study parameters, including fixation time, NBF concentration, and the site of the rat oral tissue. These effects were assessed by examining the effects of the Pan-CK and p63 immunohistochemical markers on staining measurements. The results showed that, for both markers and samples from five sites in the rat oral epithelium, the optimal fixation time was 1 day and the optimal NBF concentration was 10% (group A).

Ethical Considerations

Compliance with ethical guidelines

The protocol for handling the experimental animals was approved by the Animal Ethics Committee of the College of Dentistry, University of Baghdad (Code: 867).

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Authors' contributions

Conceptualization, resources, software, and writing the original draft: Inas Abdulridha Taha; Formal analysis, project administration, and supervision: Ban Fadhil AL-Drobie; Methodology, data curation, visualization, review and editing: All authors.

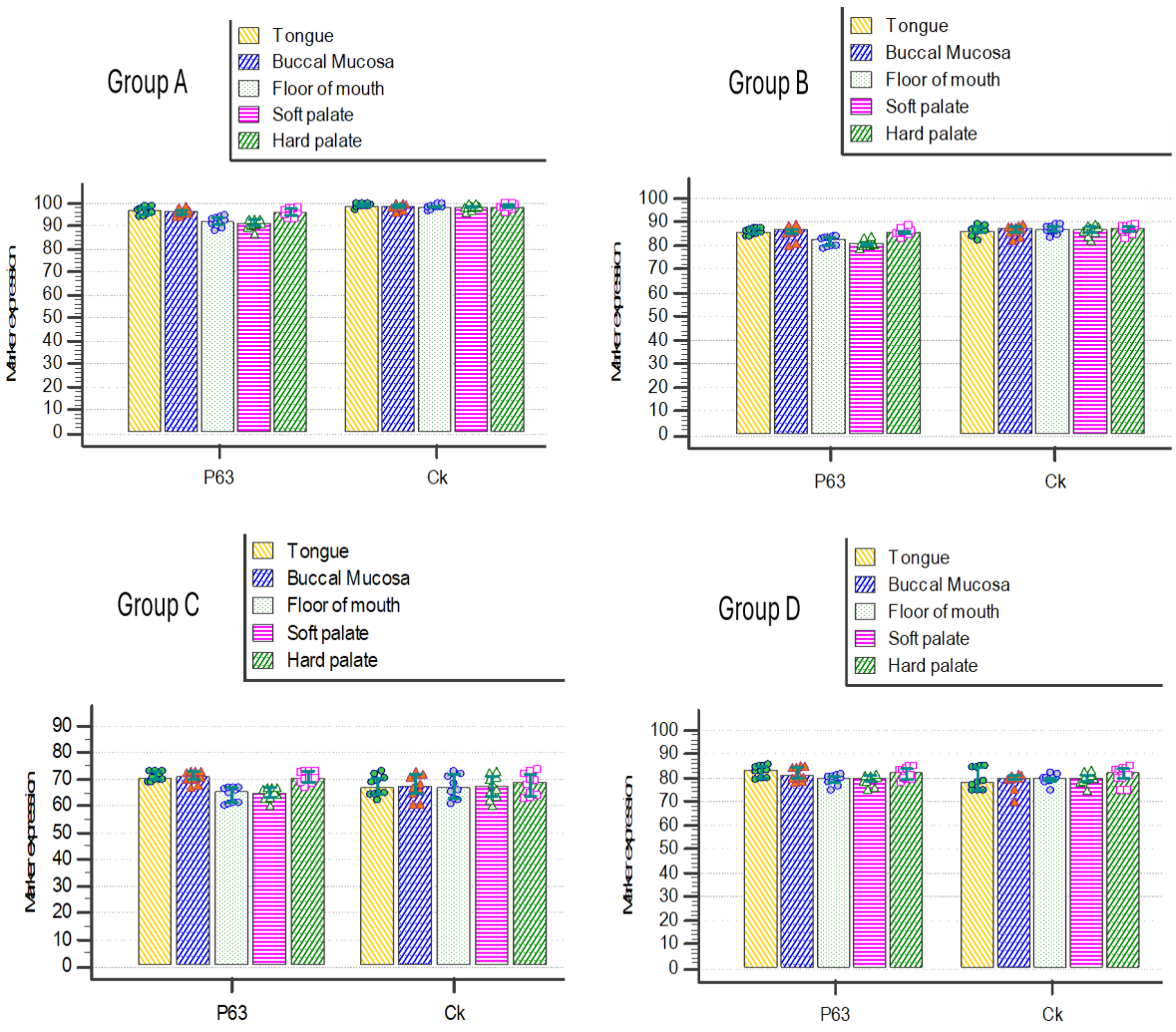


Figure 10. Median expression of Pan-CK and P63 across the five oral sites in study groups

Conflict of interest

The authors declared no conflict of interest.

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