Original Article

Cytomorphometric Evaluation of Oral Mucosa among Children undergoing Acrylic Removable Orthodontic Appliances: An in vivo Study

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Abstract

Introduction: The use of acrylic appliances in the oral cavity warrants biocompatibility evaluation. This study aimed to carry out an in vivo investigation of the cytomorphometric changes of oral mucosa among children undergoing acrylic removable orthodontic appliances. **Methods:** In this observational clinical study, acrylic removable orthodontic appliances were delivered to 25 orthodontic patients and followed for 3 months. Mucosal samples were collected by gentle brushing of the internal part of the right and left buccal mucosa before appliance delivery (T0) and 1 week (T1), 1 month (T2), and 3 months (T3) following appliance delivery. The cells were immediately prepared for cytomorphometric analysis. **Results:** There was no significant difference in the nuclear area between different time intervals. Cytoplasmic area in the right side of the buccal mucosa was significantly larger 1 month following appliance delivery (T2), compared to T0 (P=0.018) and T1 (P=0.036). On the right side, nuclear to cytoplasmic ratio values in T2 were significantly less than those in T0 (P=0.008) and T1 (P=0.002). Moreover, T3 values were lower than T1 values (P=0.048). The results of micro-nucleation and apoptosis analysis did not show statistically significant differences between different time intervals. **Conclusion:** Orthodontic acrylic appliances may not be a factor in inducing morphologic changes of oral mucosa cells; nevertheless, they may promote some transient cytometric effects. It seems that they do not cause any significant damages to the oral mucosa in long term.

Keywords: Acrylic resins, Cytotoxicity, Orthodontics

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Introduction

The use of removable acrylic appliances for prolonged duration in numerous orthodontic treatments, such as growth modification, palatal anchorage, single tooth movements, expansion, and retention, warrants special attention regarding their biocompatibility. They are constructed from acrylic resins or polymethylmethacrylates (1). Auto-polymerized resins are the most popular acrylic resins in orthodontics because of their fast and convenient application and low cost (1, 2). In acrylic resin materials, polymerization, which is the conversion of monomers into polymers, is usually incomplete. The incomplete polymerization,

especially in auto-polymerized types (3), results in a gradual release of the residual monomers, even in dry static environments. The oral environment is particularly ideal for the biodegradation of materials due to its microbiologic and enzymatic properties, the presence of saliva as an electrolyte, and fluctuations of PH and temperature (4, 5). In this regard, it is expected that materials' degradation and monomer release be accelerated in the oral cavity. Compounds, such as methyl methacrylate (MMA), in the composition of acrylic resin, have been found to be potentially allergen, toxic, and mutagenic, which can cause irritation, inflammation, and allergic reactions (1, 6, 7). Therefore, special interest is given to the biocompatibility of acrylic appliances.

Although acrylic resins have undergone developments, the biocompatibility of these materials is usually disregarded in dental practice (8). The literature includes numerous studies documenting the monomer release from acrylic orthodontic appliances in the oral cavity (9, 10). Most previous research has evaluated the effects of modes of polymerization (11), methods of acrylic manipulation (1, 3, 6, 12), procedures of polishing (3, 6), and times of water immersion (11, 13) on the amount of residual MMA. These were mainly in vitro studies (1, 6, 11, 12) or followed at most over a 7-day appliance usage with in vivo investigations (9). Ica et al. has reported that the greatest monomer release is during the first day after acrylic preparation (12). However, insufficient studies have been conducted assessing the biocompatibility of orthodontic removable appliances that could remain in contact with the oral mucosa for several months.

On the other hand, exfoliative cytology, which is a straightforward and noninvasive diagnostic method, can be considered a practical technique to evaluate the oral mucosa alterations (14, 15). Another major advance in cytopathology was the development of liquid-based cytology with more advantages over the conventional types, such as better evidence of epithelial cells, less cell overlapping, and more representative samples for reading sampling (16, 17).

There is a lack of related literature on the adverse biological effects of acrylic orthodontic appliances in vivo. Therefore, this clinical study aimed to investigate the cytomorphometric changes of the oral mucosa, including nuclear area, cytoplasmic area, nuclear to cytoplasmic ratio, and morphologic assessments, in children undergoing acrylic removable orthodontic appliances by a liquid-based exfoliative cytology method. To the best of our knowledge, this is the first in vivo study that assessed these cytomorphometric changes over 3 months.

Materials and Methods

Participants

This observational clinical study was conducted on orthodontic patients who required treatment with acrylic removable appliances. The research protocol was reviewed and approved by the Ethical Committee of Mashhad University of Medical Sciences, Mashhad, Iran (IR.MUMS.REC.1392.901). The sample size was calculated by a formula at 25 subjects using relevant studies (18, 19). Participants were selected from referring patients to the Department of Orthodontic, School of Dentistry, Mashhad University of Medical Sciences. Inclusion criteria were being within the age range of 8-13 years, requiring orthodontic therapy with maxillary removable appliance containing a posterior bite plane, having acceptable oral health, and being willing to participate in the study. On the other hand, individuals who consumed alcohol and carbonated drinks habitually, had systemic and skin diseases, used medications, had undergone previous therapy with acrylic appliances, had a history of allergic reactions, and did not cooperate were excluded from the study. Once the objectives of the research and procedures of cell collection were fully explained to patients and parents, in case of agreement, informed consent was obtained.

Acrylic appliance

Patients required a removable orthodontic acrylic appliance in the upper jaw for transverse or anterior expansion or single tooth movements according to their related orthodontic problem. The appliance components were acrylic plate with posterior bite plane, expansion screw (Dentaurum, Ispringen, Germany) or Z-spring (0.024-inch stainless steel wire, Dentaurum, Ispringen, Germany) and Adams clasps (0.028-inch stainless steel wire, Dentaurum, Ispringen, Germany) (Figure 1). The posterior bite plane was extended to the buccal surface of the posterior teeth. The thickness of the posterior bite plane was 2-3 mm. All appliances were made using autopolymerized acrylic (Vivadent/ Ivoclar AG, FL-9494 Aschaan/ Liechtenstein) by the same experienced technician who used the spray-on method (12) (the powder was saturated by the monomer). The manufacturer's instructions were closely followed for preparing the acrylic and observing powder to monomer ratio. Acrylic appliances were immersed in water for 24 h after construction. In the clinic, the appliances were adjusted in the patients' mouths. The patients were instructed to wear the appliance 24 h a day except when eating and brushing.

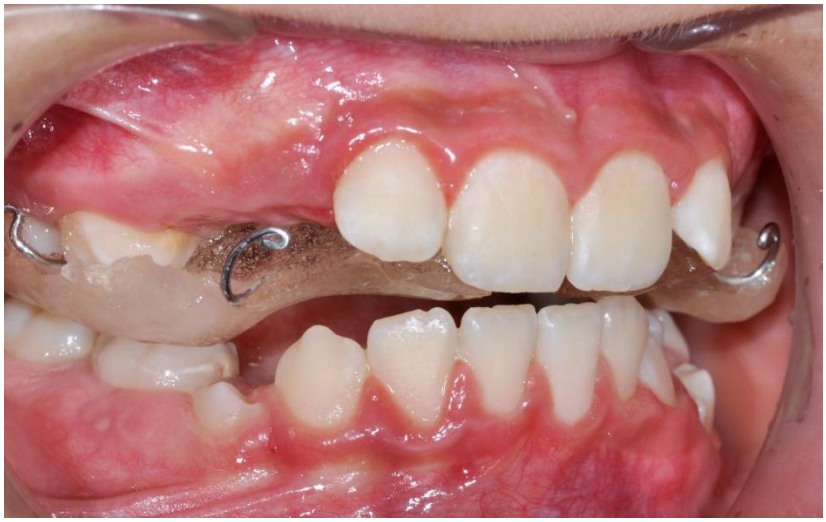

Figure 1: Intraoral lateral view of the acrylic removable orthodontic appliance

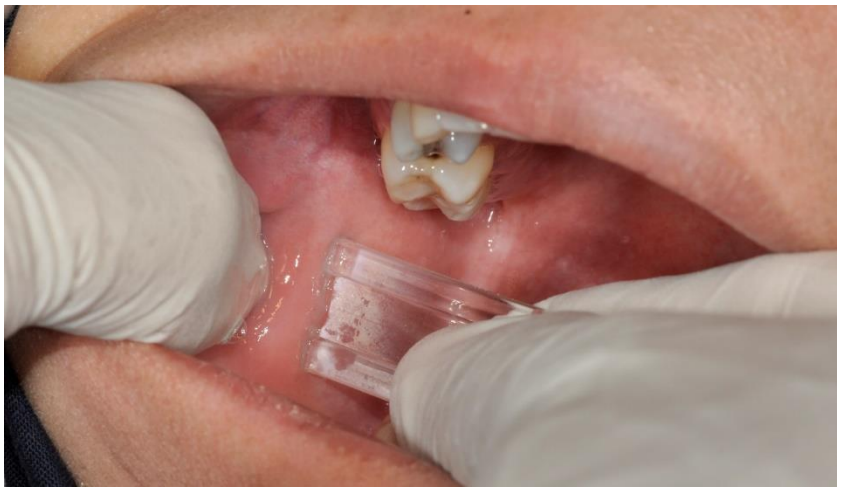

Figure 2: Oral sampling by gently scraping the buccal mucosa

Cell collection

In this liquid-based cytology study, the oral sampling site was buccal mucosal tissue. After rinsing the mouth with tap water, cells were harvested by gently scraping the right and left cheek mucosa (5, 20) with a rough blade of a plastic spatula (Figure 2). About 5-7 strokes on each side were enough to collect adequate cells. Each sample was stirred in a 5-ml tube prefilled with an alcohol-based fixative medium. The cell suspension was immediately transferred to the laboratory for cytomorphometric analysis. The samples were centrifuged at 800^{rpm} for 5 min. Following fixation in 3:1 methanol/acetic acid fixative, the samples were spread on the pre-cleaned glass slides. The slides were then stained using the Papanicolaou method.

The sampling was performed before appliance delivery to the patient (T0) and repeated 1 week (T1), 1 month (T2), and 3 months (T3) after appliance delivery. In total, eight smear samples were taken from each patient (at T0, T1, T2, and T3 from the right and left sides at each time).

Cytomorphometric assessment

An expert oral pathologist assessed the slides using a light microscope based on morphometric characteristics of the cell nucleus and cytoplasm. For cytometric assessment, nuclear area (NA), cytoplasmic area (CA), and nuclear to cytoplasmic ratio (N/C), and for morphologic assessment, micronucleus presence and apoptosis (i.e., pyknosis, karyorrhexis, and karyolysis) were measured. In each slide, 50 clearly defined cells with predominant staining were selected manually in a random fashion from different fields. In order to avoid measuring and counting the same cells again, the microscope stage was moved from left to right, and then, down and across in a stepwise manner. After determining the mean lengths of the greater and lesser diameters of each cell (considering their oval shape and taking into

account the dimensions of the nucleus), the NA, CA, and finally, N/C were calculated. Cytomorphometric analysis was carried out by transferring sample images at 100x magnification from a Leica Galen III microscope (Buffalo, New York, USA) to a computer by means of a digital camera (Sony EXWaveHAD Model No.SSCDC58AP, Tokyo, Japan). Photoshop software (version 7.0) was used for cytomorphometric assessments.

According to the design of the study, a code number was dedicated to each sample prior to their transfer to the laboratory, which was concealed from both the pathologist and the statistician. Once the statistical analysis was conducted, the numbers were decoded and the results were interpreted.

Statistical analysis

All calculations were obtained in the SPSS software (version 15). The normality of data distribution was assessed by Kolmogorov-Smirnov test. The data were presented as mean, standard deviation, median, and interquartile range (IQR). For each parameter (i.e., NA, CA, N/C, micro-nucleation, and apoptosis), Friedman's test was used to compare the values of different time intervals for the right or left side. In case of a statistically significant difference, a pairwise comparison between the four time intervals was performed using Wilcoxon Signed Rank test. The significance level was set at 0.05.

Results

A total of 25 patients participated in this clinical study. There were 9 (36%) males and 16 (64%) females with the mean age of 9.98±1.03 years (at the age range of 8.2-12.2 years). Gender differences were not considered because of the short duration of the study. All participants cooperated until the end of the study except two cases who dropped out at T3 because of lack of patient attendance. According to Kolmogorov-Smirnov test, some parameters were not distributed normally ($P<0.05$); therefore, the normal distribution hypothesis of data was rejected and nonparametric tests were used.

The results of cytomorphometric changes of oral mucosa following acrylic orthodontic appliance use are presented as follows: .

Nuclear area

Based on the results of Friedman's test, NA did not show any significant difference between different time intervals in the right (P=0.814) or left (P=0.55) side of the buccal mucosa (Table I).

Table I: Nuclear area values of the right and left buccal epithelial cells at different time intervals

	Time	n	Mean±SD	Median (IQR)	Mean rank	P-value
Right	T_0	25	1.18±0.02	1.18 (0.03)	2.28	0.814
	T_1	25	1.19±0.05	1.18 (0.05)	2.59	
	T_2	25	1.14±0.23	1.18 (0.03)	2.54	
	T_3	23	1.18±0.02	1.18 (0.02)	2.59	
Left	T_0	25	1.14±0.24	1.18 (0.04)	2.72	0.55
	T_1	25	1.13±0.23	1.18 (0.04)	2.3	
	T_2	25	1.19±0.05	1.18 (0.04)	2.65	
	T_3	23	1.18±0.03	1.18 (0.03)	2.33	

IQR: Interquartile range

Cytoplasmic area

Comparison changes of CA in the right side of the buccal mucosa revealed that there was a statistically significant difference between time intervals (P=0.01), whereas the difference of CA changes in the left side was not significant (P=0.23) (Table II). Considering the significant difference in CA values of the right side, pairwise comparisons between different times were performed. Wilcoxon Signed Rank test results showed that on the right side, the cytoplasmic area was significantly larger in T2 than in T0 (P=0.018) and T1 (P=0.036). Other pairwise comparisons did not show a

statistically significant difference (T0-T1, P=0.59; T0-T3, P=0.057; T1-T3, P=0.057; T2-T3, P=0.93)

Table II: Cytoplasmic area values of the right and left buccal epithelial cells at different time intervals

	Time	n	Mean±SD	Median (IQR)	Mean rank	P-value
Right	T_0	25	3.14±0.16	3.13 (0.22)	1.98	0.01*
	T_1	25	3.17±0.23	3.1 (0.32)	2.15	
	T_2	25	3.24±0.49	3.31 (0.39)	3.07	
	T_3	23	3.32±0.3	3.37 (0.38)	2.8	
Left	T_0	25	3.14±0.58	3.11 (0.4)	2.28	0.23
	T_1	25	3.23±0.58	3.28 (0.3)	2.54	
	T_2	25	3.32±0.21	3.35 (0.28)	2.93	
	T_3	23	3.22±0.18	3.21 (0.3)	2.24	

IQR: Interquartile range *Significant at P < 0.05

Nuclear to Cytoplasmic ratio

In cytotoxicity evaluation, the area of the N/C has particular importance; therefore, the area of the N/C was compared between different time intervals. The greatest reduction in the area of N/C in both right and left sides was found at T2 (Table III). According to Friedman's test results, there was no significant difference between different times in the left side of the buccal mucosa (P=0.29), while this difference was statistically significant on the right side (P=0.006) (Table III). Subsequently, a pairwise comparison between different times on the right side was performed using Wilcoxon Signed Rank test. Pairwise analyses on the right side showed that N/C values were significantly lower in T2 than in T0 (P=0.008) and T1 (P=0.002), and T3 values were less than T1 values (P=0.048). The differences between T0-T1, T0-T3, and T2-T3 were not statistically significant (P=0.42, P=0.053, and P=0.46, respectively).

Table III: Nuclear to cytoplasmic ratio of the right and left buccal epithelial cells at different time intervals

	Time	n	Mean±SD	Median (IQR)	Mean rank	P-value
Right	T_0	25	0.376±0.02	0.37 (0.03)	3.13	0.006*
	T_1	25	0.376±0.03	0.37 (0.03)	2.76	
	T_2	25	0.345±0.06	0.35 (0.04)	1.91	
	T_3	23	0.359±0.03	0.35 (0.05)	2.20	
Left	T_0	25	0.353±0.07	0.36 (0.05)	2.70	0.298
	T_1	25	0.343±0.07	0.35 (0.03)	2.48	
	T_2	25	0.358±0.02	0.35 (0.04)	2.09	
	T_3	23	0.365±0.02	0.37 (0.03)	2.74	

IQR: Interquartile range

Morphologic changes

Mutagenicity of cells, which is assessed based on micro-nucleation and apoptosis (i.e., pyknosis, karyorrhexis, and karyolysis) of cells, was compared at different time intervals. According to Friedman's test, neither the micro-nucleation nor the apoptosis results showed a statistically significant difference between different times in the right and left buccal mucosa (tables IV and V).

Table IV: Micro-nucleation values of the right and left buccal epithelial cells at different time intervals

	Time	n	Mean±SD	Median (IQR)	Mean rank	P-value
Right	T_0	14	8±4.5	8 (7)	2.67	0.801
	T_1	15	7.26±4.84	6 (9)	2.33	
	T_2	20	9.5±7.43	7 (8.75)	2	
	T_3	16	9.125±4.88	7.5 (9.5)	3	
Left	T_0	15	6.666±4.43	5 (5)	1.6	0.178
	T_1	14	9.428±4.53	8 (5.75)	2.6	
	T_2	20	8.2±4.71	7.5 (4)	2.4	
	T_3	15	8.866±4.29	9 (7)	3.4	

IQR: Interquartile range

Table V: Apoptosis values of the right and left buccal epithelial cells at different time intervals

	Time	n	Mean±SD	Median (IQR)	Mean rank	P-value
Right	T_0	14	7.571±8.39	5.5 (4.75)	1.67	0.102
	T_1	15	7.066±3.8	7 (6)	2.33	
	T_2	20	7.5±4.86	7 (8.25)	4	
	T_3	16	8.062±6.73	5.5 (5.5)	2	
Left	T_0	15	5.066±3.05	4 (4)	1.92	0.337
	T_1	14	6.857±4.18	6.5 (2.5)	2.92	
	T_2	20	6.45±3.64	5 (7)	3	
	T_3	14	5.357±2.79	4.5 (4.25)	2.17	

IQR: Interquartile range

Discussion

This study evaluated the biocompatibility of acrylic resin-based removable orthodontic appliances in the oral cavity over 3 months. Laboratory influential factors, such as the type of polymerization, brand of acrylic powder and monomer, duration of water immersion, and method of acryl preparation, were homogenized for all patients as much as possible by training an expert technician.

To the best of our knowledge, no clinical study has been dedicated to evaluating the biocompatibility of acrylic orthodontic appliances for 3 months and the majority of previously conducted studies were in-vitro. Harmful factors, including the released monomers from acrylic

resins, could cause some changes, such as micronucleus formation in the basal cell layer of the epithelium. Basal cells constitute the lowest layer of epithelium in which mitosis is happening continuously. Due to this rapid turnover rate, within 25 days, epithelial cells emerge to the surface and exfoliate (21, 22). For this reason, the samples in the current study were collected just before and 7 days after appliance delivery to understand the immediate effect of acrylic resin appliances. Moreover, to monitor the influences over a longer duration of time and perhaps the additive effects of chewing, sampling was repeated 1 and 3 months after appliance delivery.

According to the results, most cytomorphometric evaluations did not show significant differences between before (T0) and after appliance delivery. However, there were significant differences in CA and N/C between the beginning of the study (T0) and 7 days (T1) and 1 month after appliance delivery (T2). Moreover, the difference in N/C between 7 days and 3 months after appliance delivery (T3) was statistically significant. It should again be noted that these differences were only seen in the rightbuccal mucosa in the CA and area of the N/C. The other evaluations of NA, micro-nucleation, and apoptosis of cells in the right side and all the evaluations of the left buccal mucosa showed nonsignificant differences between before appliance delivery and after different time intervals. Although monomer release has been reported starting as early as 2 h after appliance construction to 3 months later (12), it appears that the cytomorphometric changes are mild and should not be a cause for concern. However, our literature review has failed to find a similar clinical study that has evaluated the cytotoxicity of acrylic appliances for 3 months or more; therefore, comparing our findings with other studies is limited and difficult. The reason behind the relative safe behavior of acrylic appliances in this study might be explained by the defense mechanism of the body, such as antioxidant properties of saliva (23), and the low release of monomer over time.

It has been reported that monomer release is high in the first few hours after acrylic preparation and is reduced after 24 h (12). In in-vitro studies, tests and evaluations can be repeated multiple times without limitations. In contrast, due to the clinical nature and associated ethical issues of the current in vivo study, sampling could not be repeated at multiple short intervals such as 1, 12, 24, and 48 h following appliance delivery. Moreover, acrylic appliances could not be delivered to the patients immediately after construction due to ethical issues; in this regard, it is impossible to make judgments or comments on the side effects of acrylic appliances during the first few hours after construction.

Orthodontic removable appliances are kept in contact with the oral mucosa for a long period of time; consequently, it is desirable to reduce the residual monomer content as much as possible before they are placed in the mouth (1, 12). In this study, the appliances were immersed in water for 24 h according to previous recommendations (9, 11, 24). The reason for this measure is that most residual monomer is released during the first day following appliance construction (12). The process of water immersion may have contributed to the relatively safe behavior of acrylic appliances in this study. The 24-hour immersion of acrylic appliances may not always be carried out due to time shortages. It is important that this precaution is followed by laboratory technicians and emphasized by the prescribing orthodontists.

According to the results, some cytomorphometric changes were statistically significant in the right buccal mucosa, compared to the left side. These differences might be attributed to the habit of chewing of patients on one side. However, the researchers of the current study lacked any information about the chewing habits and patterns of the studied patients. The degradation process of materials in the mouth is dependent on the fatigue following repetitive loads, such as masticatory forces (19, 25). Nevertheless, there is controversy surrounding the effects of chewing forces on the leakage of materials in the oral cavity. Jones et al. (26) demonstrated that the effect of chewing simulations on the leakage of soft polymers was not significant. Nevertheless, Graham et al. showed that the leach of plasticizers from denture liners was much higher in the mouth than in the laboratory environment (27).

To the best of our knowledge, there are few studies on the antioxidant defense system of saliva (28, 29, 30). Antioxidants protect against the potentially harmful effects of processes or reactions that cause excessive oxidation (23). The amounts of leaching monomer causing toxicity are not well understood, and it is difficult to truly assess the levels that induce cellular damage. Furthermore, the effects of different foods and drinks on the biodegradation of acrylic appliances are questionable.

The strengths of the current study were its clinical nature of the research, relatively long duration of follow-up, and reasonable sample size. However, there were a number of limitations to our study. There was no control on different diets and eating habits of participants, and there were restrictions in repeating sampling at short intervals. There are several gaps in the literature regarding the cytotoxicity of acrylic orthodontic removable appliances. Therefore, for future research, it is recommended that the cytotoxicity of acrylic appliances be evaluated over

longer periods of time. Evaluation of the biocompatibility of different acrylic resins brands can also benefit from further research.

Conclusion

While recognizing the limitations of our study, it can be concluded that if acrylic orthodontic removable appliances are prepared in a standard manner and immersed for 24 h in the water following construction, their application may not be a factor in inducing morphologic changes in oral mucosa cells; nonetheless, it may promote some transient cytometric effects. However, it appears that these changes do not cause significant damage to the oral mucosal epithelial cells in long term, and therefore, are relatively safe.

Conflicts of interest

The authors declare that there is no conflict of interest.

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