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Micronuclei and Cytomorphometric Alterations as Early Biomarkers of Mucosal Stress in Fixed (Metal/Ceramic) and Removable Orthodontic Therapy: A Prospective Comparative Study

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Abstract

Background

Orthodontic appliances may induce mechanical and biochemical stress on the oral mucosa, potentially leading to early cytological alterations.

Objective

To evaluate and compare the cytomorphometric parameters and micronuclei (MN) frequency in fixed metal, ceramic, and removable orthodontic appliances in adolescents at 1- and 6-weeks post-treatment.

Methods

A prospective study was conducted on 30 adolescents divided into three groups: metal, ceramic, and removal orthodontic appliance (n = 10 each). Exfoliative cytology using Papanicolaou staining was performed at two experimental sites (S1, S2) and one control site (S3) at 1 week (T1) and 6 weeks (T2). Intergroup comparisons were made using Kruskal–Wallis, and the Wilcoxon signed-rank test was used for within-group comparisons.

Results

Intergroup comparison showed no significant differences for cytoplasmic (C) area and nuclear (N) area, and N:C ratio across most comparisons ($p > 0.05$) ($p = 0.096–0.995$; $\eta^2 = 0.0003–0.16$). A difference was noted only for MN frequency at the S1T1 ($p = 0.04$; $\eta^2=0.22$). Intragroup analysis showed that a significant increase was seen in MN ($p \leq 0.028$) and N:C ratio ($p = 0.002$) across all sites (S1, S2, S3) with strong effect sizes, in the fixed metal appliance group. The ceramic appliance group showed limited, site-specific changes, with significance mainly in N:C ratio at S3 and nuclear area ($p \leq 0.037$). The removable appliance group showed a significant increase in MN, N:C ratio, and cytoplasmic area across selected sites, with strong effect sizes.

Conclusion

Orthodontic treatment is associated with cytological alterations in the oral mucosa in early stages, with MN and N:C ratio acting as sensitive and reliable biomarkers of appliance-induced cytological alterations suggestive of cytogenic stress, especially in fixed metal and removable orthodontic appliance systems.

Introduction

Orthodontic appliances, both fixed and removable, are widely used for the correction of dentofacial anomalies. However, their placement in healthy patients may promote dental plaque accumulation and act as a mucosal irritant due to frictional contact with the appliances. This may alter the normal oral microbiome homeostasis, cause infections and lesions in the periodontium or buccal mucosa, which are usually seen early in the adaptive phase of the orthodontic treatment.(1) Additional situations, like an ill-fitting removable orthodontic appliance or a poking wire in a fixed orthodontic appliance, may further cause trauma to the adjacent soft tissues and result in pain and discomfort to the patient.(2) The cellular changes occurring in the oral mucosa, as a result, can be detected very early by a non-invasive convenient method of oral exfoliative cytology, which is explored routinely for oral malignant and pre-malignant lesions. (1)Besides, prolonged placement of orthodontic appliances in the oral cavity is shown to cause the release of metallic ions such as nickel, chromium, and cobalt(3,4), which is a matter of grave concern clinically, as metallic ions have been associated with genotoxicity by known

effects of oxidative DNA damage or interference with DNA replication.(5) Hence, periodic monitoring of oral biofluids and mucosal cellular analysis may be necessary for the assessment of genetic damage, which can be assessed by various cytological changes in oral cells, including the presence of micronuclei (MN), pyknosis, karyolysis, karyorrhexis, binucleation, etc.

Thus, early detection of such cellular alterations is essential for ensuring the biological safety of orthodontic treatment. Oral exfoliative cytology provides a non-invasive and reliable method for assessing epithelial cell changes and has been extensively used in the detection of oral premalignant and malignant conditions. This technique, though subjective, has shown promising results in current times, as it has the possibility of complementing other laboratory techniques of cytomorphology, molecular biology, and immunohistochemistry. (1) Among cytological biomarkers, micronuclei (MN) are considered sensitive indicators of chromosomal damage and genotoxic stress. Cytomorphometric parameters such as cytoplasmic area, nuclear area, and nuclear-to-cytoplasmic (N:C) ratio reflect cellular activity and maturation status. (6)

However, limited evidence exists comparing these parameters across different orthodontic appliance systems (1,4), particularly removable appliances (3), which are widely used in developing countries like India. Furthermore, considering the prolonged duration of orthodontic treatment, there is a critical need for robust, high-quality evidence to establish whether both fixed and removable appliances are associated with any significant alterations in the cellular morphology of the oral mucosa.

Hence, the current study intends to examine the cytomorphological changes associated with removable, metal and ceramic fixed orthodontic appliances, at specific sites in contact with the appliances, and compare with the control sites at different observation times.

Materials and Methods

Study Design and Ethical Approval

This prospective, non-randomized comparative clinical study was conducted following approval from the Institutional Ethics Committee (10/3/369/JMI/IEC/2022). All participants and their guardians gave a written informed consent before enrollment.

Study Population and Sample Allocation

Thirty adolescent patients (aged 10–19 years) reporting to the orthodontic OPD for treatment at a tertiary dental care institute in India were recruited.

Participants were purposively sampled based on the eligibility criteria and assigned to the three groups. The sample size used in the current study was comparable to earlier orthodontic studies on cytomorphometric changes that have reported statistically significant findings with smaller cohorts. (1,5) The allocation of participants was done non-randomly into the three groups based on the orthodontic appliance included. Group A: Fixed orthodontic metal appliances, Group B: Fixed orthodontic ceramic appliances, and Group C: Removable orthodontic appliances. *Inclusion criteria:* Good oral hygiene, no active carious lesions, absence of oral mucosal disease, and no harmful oral habits.

Exclusion criteria: Patient with history of systemic illness, tobacco or alcohol abuse, or recent antiseptic mouthwashes use. This was important to remove any effect on cellular characteristics of mucosa.

Sample Size Calculation

The sample size was determined using a pre-study power analysis assessing the mean difference in the nuclear area for one-way analysis of variance (ANOVA), based on a previous study.(5) A sample size of 30 (10 per group) was calculated using an effect size (f) of 0.59, at 5% level of significance and 80% power, using G*Power. Although the sample size was based on prior orthodontic literature, the small cohort of 10 participants per group interprets the study as exploratory.

Orthodontic Appliance Protocol

The metal and ceramic fixed orthodontic treatment in Groups A and B respectively were treated using a straight-wire MBT prescription system (0.022" × 0.028"). Preliminary arch alignment was done using 0.012" nickel–titanium (NiTi) archwires. (Ortho Organizers, USA).

Removable maxillary Hawley's appliance was delivered to patients in Group C. It consisted of a labial bow and Adam's clasp fabricated from 0.7" stainless steel wire, with an acrylic base plate made from clear cold-cure polymethyl methacrylate resin (DPI, Mumbai, India).

Sampling Sites and Study Timeline (CONSORT style workflow flowchart adapted for non-randomized study, depicted in Fig 2)

A single operator (PS) took the oral cytological smears from three predefined sites under the supervision of PK. The sites for collection of smears were divided into experimental (S1 and S2) and control (S3) sites. The sites in direct contact with orthodontic appliance components were experimental and the sites in the mucosa not in direct contact with any appliance component were control sites. They were different from fixed orthodontic and removable appliances groups. (Fig 1a represents the sites in Groups A and B, while Fig 1b represents the sites in Group C)

Sampling was performed at two clinically relevant time points:

T1: 1 week after appliance placement, representing the early adaptation phase

T2: 6 weeks after treatment initiation, representing a stabilized adaptation phase

No true T0 (pretreatment) baseline, so control sites were sampled within the same post-placement frame. This limits the ability to distinguish pre-existing variation from treatment-associated changes

At each time point, three samples were obtained per patient, resulting in six samples per patient across both time points. Accordingly, for 30 patients (10 per group), a total of 180 cytological samples were collected and analyzed. Fig 3 depicts the armamentarium for clinical workflow and cytological sampling methodology.

Collection and fixation of Cytological Smears

Before taking the smear (sample), the chosen oral mucosal site was carefully cleansed for removal of debris and saliva. To minimise stress and cellular distortion, superficial epithelial cells were obtained by gently scraping them with a sterile wooden spatula soaked in normal saline under consistent pressure. To prevent overlap or clumping, the collected material was quickly applied as a thin, even smear onto a clean, labelled glass slide. To maintain cellular morphology, slides were quickly fixed in 95% ethanol and spray fixative. Then, under the guidance of an experienced oral pathologist (AC), they were stained using the conventional Papanicolaou (PAP) method for ensuring uniformity and quality control. (7–9)

Microscopic Evaluation and Cytomorphometric Analysis

A binocular research microscope (OLYMPUS, Noida, India) equipped with specialised imaging and analysis software (Magnus Pro) was used to capture and evaluate each PAP-stained smear. An HP Pavilion Full HD monitor (1920 × 1080) with uniform brightness (60–70%), default contrast, and controlled ambient lighting was used for digital picture processing. Blue-light filters and auto-brightness were turned off. It took eight to twelve minutes to fully evaluate each slide. To reduce eye strain, observer tiredness, and scoring bias, slides were inspected in batches of five to six, with intervals of five to ten minutes. Systematic zigzag scanning was used to assess at least 100–200 distinct epithelial cells on each slide, with a 10X initial screening and a 40X comprehensive evaluation, done by experienced oral pathologist (AD). Distorted or overlapping cells were not included. Nuclear area, cytoplasmic area, and nuclear-to-cytoplasmic (N: C) ratio were measured from 100 typical cells as part of the cytomorphometric evaluation. The following criteria were used to identify micronuclei: they had a smooth,

rounded periphery, stained similarly to the nucleus, had a diameter of between 1/3 and 1/16 of the main nucleus, and were clearly separated from it. (Representative images are shown in Fig. 4a and Fig. 4b). The total MN count per cell assessed was used to calculate the mean micronuclei frequency. Parameters assessed are summarised in Table 1. (10–12)

Table 1. Represents the parameters evaluated in the smears and their outcome measures

Parameter	Method of Evaluation	Cells Counted	Outcome Measure
Cellular adequacy	Microscopic screening	Entire smear	Presence of well-defined epithelial cells
Cytoplasmic area	Image analysis software	100 cells	Mean cytoplasmic area in square micrometers (μm²)
Nuclear area	Image analysis software	100 cells	Mean nuclear area in square micrometers (μm²).
N:C ratio	Derived from measurements	100 cells	Mean nuclear-cytoplasmic ratio
Micronuclei frequency	Morphological criteria-based counting	100–200 cells	Mean MN frequency

Statistical Analysis

Jamovi (version 2.7.15) was used for data analysis. Shapiro–Wilk test was used to assess the normality of data distribution. Sample size estimation, though, was based on parametric assumptions for ANOVA; the final evaluation used non-parametric tests because the Shapiro–Wilk test showed deviation from normality in several post-treatment and time-difference (T2–T1) variables. This approach was adopted to ensure appropriate analysis of the observed data distribution; however, the shift from parametric planning to non-parametric testing may affect statistical power and should be considered when interpreting the findings. Median and Interquartile ranges were reported for all variables. Kruskal–Wallis test was used for intergroup comparisons, and the Wilcoxon signed-rank test was used for intragroup comparisons. Statistical significance was set at $p<0.05$.

Results

Intergroup Comparison (Kruskal–Wallis Test) (Table 2)

Intergroup comparison across Groups A, B, and C showed no statistically significant differences for cytoplasmic area, nuclear area, and N:C ratio at most sites and time points (p-values ranging from 0.096-0.995, effect size $\eta^2 = 0.0003$-0.16). A statistically significant difference was observed only for micronuclei at S1 T1 ($p = 0.04$, $\eta^2 = 0.22$). However, since T1 depicts measurements a week after treatment start rather than a true baseline, this outcome may reflect pre-existing variability rather than a treatment effect. However, subsequent comparisons for micronuclei were non-significant ($p > 0.05$, $\eta^2 = 0.05$–0.11). Overall, the effect sizes were predominantly small, indicating limited between-group variability across study parameters.

Intragroup Comparison at different time points (Table 3-5)

Group A (Fixed orthodontic metal appliance) (Table 3)

Intragroup analysis revealed no statistically significant changes in cytoplasmic area across all sites ($p = 0.084$-1.0). Micronuclei frequency increased significantly at all sites (S1, S2, S3; $p = 0.008$, 0.028, and 0.027, respectively), with large effect sizes. The N:C ratio also showed statistically significant increase across all sites ($p = 0.002$), with consistently large effect sizes. Nuclear area showed partial significance, at S2 ($p = 0.002$) and S3 ($p = 0.049$), while S1 remained non-significant ($p = 0.064$). Overall, Group A showed consistent time-dependent cytological alterations, particularly in MN and N:C ratio.

Group B (Fixed orthodontic ceramic appliance) (Table 4)

Cytoplasmic area did not show statistically significant changes across all sites (p = 0.232-0.846). Micronuclei demonstrated a significant increase only at S1 (p = 0.014), while S2 and S3 remained non-significant. The N:C ratio showed statistical significance only at S3 (p = 0.037), and the nuclear area was significant only at S3 (p = 0.002). Overall, Group B exhibited limited and site-specific cytological changes.

Group C (Removable orthodontic appliance) (Table 5)

Cytoplasmic area showed significant increases at S2 and S3 (p = 0.002 and 0.009 respectively), while S1 remained non-significant (p = 0.084). Micronuclei frequency increased significantly across all sites (p = 0.024, 0.014, and 0.006), with large effect sizes. The N:C ratio also showed highly significant increases across all sites (p = 0.002). Overall, Group C showed time-dependent cytological changes across multiple parameters.

Discussion

The present study evaluated early cytological and cytomorphometric changes in the oral mucosa associated with different orthodontic appliances. The findings suggest time-dependent epithelial alterations associated with orthodontic treatment, particularly in micronuclei (MN) frequency and nuclear-to-cytoplasmic (N:C) ratio. These findings are consistent with the literature reporting cytogenetic alterations and associated biological responses consequent to orthodontic therapy in oral exfoliated cells. (1,4,14)

The intergroup comparisons showed no significant differences among the three groups across nearly all parameters (approximately 95–97%). The micronuclei at S1T1 were the only statistically significant parameter, which more likely reflects initial heterogeneity among groups than a treatment effect. The intergroup analyses further revealed small effect sizes, thus supporting the comparable outcomes of the three different interventions. Accordingly, the findings do not support strong claims of differential biological harm among the appliance types.

In contrast, intragroup comparisons documented time-dependent distinct and clinically meaningful changes. An increase in MN frequency was observed across different time points within groups, especially in the fixed metal and removable appliance cohorts. MN is widely recognized as a sensitive biomarker of cytogenetic alteration; however, its presence alone does not establish direct DNA damage or irreversible genotoxic effects in oral exfoliated cells. (14,19) Such changes may reflect transitory epithelial stress associated with orthodontic treatment due to mechanical irritation, exposure to orthodontic materials, or low-grade inflammatory responses rather than irreversible genomic injury. (1,13,17) However, since a true pre-treatment baseline (T0) was not taken, these changes may be attributed to temporal associations rather than as direct causal genotoxic effects.

An additional observation was the increase in MN at the non-contact control site (S3). This may suggest a broader epithelial or inflammatory response rather than a restricted mucosal response at the direct appliance-contact areas during orthodontic treatment. (21,22) Similar field-like responses have been advocated in literature, potentially facilitated by oxidative stress or inflammatory signalling. (17,21,22). Nevertheless, this explanation remains hypothetical due to the absence of biochemical or molecular markers and thus cannot be verified.

In total, Groups A and C showed an increase in micronuclei and N:C ratio reflecting time-dependent cytological changes linked to treatment-induced stress or adaptive remodeling. Similarly, alteration in the nuclear area indicates cellular modifications following intervention. The cytoplasmic area, however, showed no significant changes across most groups, suggesting that nuclear alterations, as compared to cytoplasmic parameters, may be more sensitive indicators of treatment response.

Thus, the findings show that there was no significant difference between the three different groups; they produced measurable time-bound cytological changes within the groups. Within this cohort, Group C demonstrated relatively more consistent within-group changes, followed by Group A. However, these observations should be interpreted cautiously, given

the largely non-significant intergroup comparisons. These results bring forth the importance of intragroup longitudinal analysis for detecting treatment-related changes which are not apparent through intergroup comparisons alone.

Metal versus Removable Appliances: Early Cytological Changes

The micronucleus (MN) frequency at S1–T1 was significantly higher in the Group A metal appliance group ($p = 0.011$), showing an early localized cytological change at the S1 site where direct bracket–mucosa interaction occurred. Current evidence, however, shows that fixed orthodontic therapy is known to cause early cytogenetic alterations in buccal epithelial cells, specifically during the initial treatment phases, but these effects often lessen over time.(13–15) Since our study is just 6 weeks into treatment, further research can be initiated for a longer duration to assess the changes over time in all three groups.

There was a progressive increase in MN frequency seen across S1, S2, and S3. This indicates that the detected cytological changes suggestive of cytogenetic stress may reflect a broader mucosal response beyond localized mechanical irritation. This involves the release of metal ions(nickel, chromium), coupled with oxidative stress, and inflammatory pathways, as shown in previous studies.(16,17) However, these mechanisms could not be confirmed in the current study due to a lack of molecular or biochemical markers.

In contrast, the removable appliance group demonstrated no significant differences in early stages but a progressive rise in MN frequency over time ($p < 0.01–0.001$). This indicated a gradual cytological response, which may be due to an increased mucosal contact with acrylic resins and appliance components. Current evidence indicates that dental materials like acrylic may initiate mild cytotoxic and genotoxic effects when exposed for a prolonged duration, although the changes may be reversible after appliance removal.(18)

Importantly, current literature highlights the role of MN as a sensitive biomarker of early cytological changes suggestive of genotoxic stress (15,19) but MN findings alone do not establish irreversible or clinically significant epithelial alterations, particularly due to the high turnover and repair capacity of oral epithelium.

Cytomorphometric Alterations and Adaptive Responses

Cytomorphometric analysis showed no significant differences in cytoplasmic area between the groups, suggesting relative stability in overall epithelial cell size.

However, the removable appliance group showed a significant rise in nuclear area and N:C ratio with time ($p = 0.008–0.047$). This suggests a time-dependent cellular adaptive response, which includes a change in proliferation dynamics and delayed epithelial maturation. Such nuclear changes may be seen as indicators of adaptive epithelial responses to low-grade or prolonged stress rather than direct genotoxic injury. (17,19) The relative stability of cytoplasmic measurements, in contrast with the more receptive nuclear parameters, suggests that nuclear indices may be more sensitive indicators of early epithelial change during orthodontic treatment. (6,10)

In contrast, the metal appliance group showed negligible cytomorphometric alterations, although it demonstrated higher early MN frequency. This suggests a predominantly cytological stress-related response in this group rather than purely morphometric.

Conceptual Biomarker Model: Ortho-Monitor Framework (Fig. 5)

The findings of the current study are consistent with a conceptual, hypothesis-generating dual-signal biomarker model:

- **Signal 1 (Cytological)**: Early epithelial alterations reflected by an increase in MN frequency across sites and time points.
- **Signal 2 (Cytomorphometric/Adaptive)**: Compensatory cellular adaptation shown by changes in nuclear area and N/C ratio, specifically in the removable appliance group.

This dual-pathway model is hypothesis-generating and requires validation. It is in alignment with the current evidence that suggests a low-grade, transient epithelial changes induced by orthodontic treatment rather than persistent genotoxicity. The outcomes are further influenced by material composition and exposure duration.(15,17,19)

Furthermore, many studies in current literature suggest the MN assay has been reaffirmed in recent studies as a reliable, non-invasive bio-monitoring tool for identifying initial cytogenetic damage in oral epithelial cells. (2,19)

The present study has several important limitations. The short duration of study (6 weeks) and the absence of a true pre-treatment baseline (T0) limit the ability to distinguish pre-existing variation from treatment-associated changes. In addition, sample size estimation was based on parametric ANOVA assumptions, whereas the final analysis used non-parametric tests because several variables deviated from normality; this should be considered when interpreting power and effect estimates. The non-randomised design and purposive sampling may have introduced the risk of confounding, as parameters such as age, sex, oral hygiene status, and baseline mucosal characteristics were not controlled for in the analysis. Despite these concerns, the study reports preliminary evidence of site-specific and patient-specific cytological responses associated with orthodontic treatment.

In conclusion, within the limitations of this exploratory study, orthodontic appliance therapy was associated with time-dependent alterations in the cytological parameters of oral mucosa, particularly MN and N:C ratio. (14,20,23). These observations support the role of non-invasive exfoliative cytology as an additional tool for monitoring initial epithelial changes during orthodontic treatment. However, the increase in MN should be studied as evidence of cytological alteration suggestive of genotoxic stress rather than irreversible genotoxic damage associated with treatment. (14,19,23). Further well-designed longitudinal studies with larger samples and a true baseline are required before drawing stronger conclusions.

Conclusions

- Within the limitations of the current exploratory study, orthodontic appliance therapy was associated with time-dependent alterations in oral mucosal cells, evident from changes between T1 and T2 across groups.
- The most consistent and sensitive indicators of cellular alterations were micronuclei frequency (MN) and nuclear-to-cytoplasmic (N:C) ratio.
- Intragroup changes, particularly in fixed metal and removable orthodontic appliances showed more pronounced and consistent findings, while ceramic appliances group showed limited and site-specific variations.
- Intergroup differences are mostly non-significant, with small effect sizes, representing nominal clinical distinction between different appliance types, despite noticeable cytological changes.
- The observed increase in micronuclei frequency suggests cytological stress and possible genotoxic change, stressing their potential role as biomarkers for observing mucosal response during orthodontic treatment.

Clinical implications

The observation that MN frequency increases significantly even at non-contact control sites (S3) suggests that orthodontic-associated mucosal changes may extend beyond the immediate appliance–tissue interface. The hypothesis generated indicates a broader field-like epithelial response rather than purely localized mechanical irritation. However, this interpretation remains hypothesis-generating, as the present study did not study any biochemical or molecular markers. (20,21).

These findings supports non-invasive biomonitoring approaches in routine orthodontic care, particularly during the early phases of treatment when cytological changes are most pronounced. The micronucleus assay, being simple, cost-effective, and minimally invasive, has been widely validated as a reliable tool for detecting early cytological changes in exfoliated buccal epithelial cells.(2,22)

Integration of MN-based screening into chairside cytological workflows could enable early identification of patients exhibiting higher-than-expected genotoxic responses despite optimal appliance design and oral hygiene maintenance. (23) Such an approach may be particularly valuable in pediatric and adolescent populations, where removable appliances are commonly used as first-line treatment modalities, especially in resource-limited settings.

In the long term, combining serial MN and cytomorphometric assessments with molecular biomarkers such as microRNAs and oxidative stress indicators could enhance the sensitivity and specificity of biological monitoring.(24) This integrated approach may facilitate the development of a personalized, risk-adaptive orthodontic framework, enabling clinicians to tailor treatment based on individual biological responses.

Such a model aligns with emerging concepts of precision dentistry and immuno-orthodontics, where treatment planning incorporates patient-specific inflammatory, genetic, and adaptive responses to optimize outcomes and improve safety. (20,22).

Future scope

Future studies should incorporate MN assessment to better understand the biological mechanisms during orthodontic treatment, along with additional molecular markers, such as oxidative stress indicators and microRNA profiles, This approach may improve the sensitivity and interpretability of bio-monitoring.

Further research should be carried out in larger longitudinal cohorts with a true baseline and multiple follow-up points, including the retention phase, to determine whether the observed changes are transient or persistent. This would help distinguish reversible adaptive changes from sustained cellular responses.

The proposed Ortho-Monitor Framework should be regarded as a conceptual model that requires further validation before clinical application. Refinement of this framework with molecular and bioinformatics approaches may contribute to the development of a multi-level biomarker model in precision-oriented orthodontic care.

Abbreviations

ANOVA — Analysis of Variance

CONSORT — Consolidated Standards of Reporting Trials

DNA — Deoxyribonucleic Acid

IQR — Interquartile Range

MBT — McLaughlin-Bennett-Trevisi

MN — Micronuclei

N:C — Nuclear-to-Cytoplasmic ratio

NiTi — Nickel-Titanium

PAP — Papanicolaou

Declarations

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Tables

Table 2: Intergroup Comparison Using Kruskal–Wallis Test

Parameter	Time point	Group A Median (IQR)	Group B Median (IQR)	Group C Median (IQR)	p-value	Effect size (η^2)	Interpretation
Cytoplasm Area	S1 T1	2331 (2240–2685)	2296 (2140–2490)	1993 (1815–2393)	0.144	0.13	NS
	S1 T2	2437 (2069–2754)	2483 (2320–2738)	2017 (1806–2412)	0.096	0.16	NS
	S2 T1	2332 (1855–2626)	2319 (1829–2612)	2333 (1889–2460)	0.924	0.005	NS
	S2 T2	2175 (1931–2327)	2151 (1936–2334)	2363 (1901–2486)	0.602	0.035	NS
	S3 T1	2126 (1946–2381)	2131 (1953–2321)	2209 (1906–2548)	0.995	0.0003	NS
	S3 T2	2056 (1983–2196)	2042 (1970–2213)	2357 (1938–2574)	0.779	0.017	NS
Micronuclei	S1 T1	7 (5.25–8)	5.5 (4.25–6)	4.5 (4–6)	0.04	**0.22**	**Significant**
	S1 T2	9 (7.25–11.75)	8.5 (6–10)	6 (5–9.25)	0.184	0.11	NS
	S2 T1	6.5 (5.25–7)	4.5 (3.25–6)	5 (4.25–6)	0.222	0.10	NS
	S2 T2	8 (7–10)	6.5 (6–8.75)	8.5 (7.25–9.75)	0.422	0.05	NS
	S3 T1	6 (4–6.75)	5 (2–5)	4.5 (4–6.75)	0.403	0.06	NS
	S3 T2	7 (5.5–9)	6.5 (4.5–7.75)	8.5 (6.5–9.75)	0.271	0.09	NS
N:C Ratio	S1 T1	0.03445 (0.03156-0.04378)	0.03366 (0.03005-0.04383)	0.03682 (0.029250.04321)	0.922	0.005	NS
	S1 T2	0.03563 (0.03307-0.04521)	0.0342 (0.02803-0.04176)	0.03894 (0.03251-0.04542)	0.437	0.057	NS
	S2 T1	0.03234 (0.02877-0.03872)	0.03344 (0.02629-0.03756)	0.03761 (0.0273-0.04463)	0.420	0.059	NS
	S2 T2	0.03993 (0.03574-0.04487)	0.03504 (0.02851-0.04301)	0.03825 (0.02835-0.04507)	0.561	0.039	NS
	S3 T1	0.03609 (0.02905-0.04578)	0.03574 (0.02877-0.04516)	0.03902 (0.03445-0.04388)	0.804	0.015	NS
	S3 T2	0.03862 (0.03441-0.04647)	0.03769 (0.03414-0.04664)	0.04099 (0.03518-0.0473)	0.976	0.001	NS

Nuclear Area	S1 T1	80.98975 (77.7272-95.07375)	76.8485 (71.09415-87.11525)	77.332 (66.49363-87.88612)	0.364	0.069	NS
	S1 T2	82.3105 (79.49213-100.78975)	79.8105 (71.15363-113.04)	81.3365 (76.71438-93.7715)	0.756	0.019	NS
	S2 T1	70.65975 (60.3295-88.245)	63.312 (54.19363-83.745)	82.086 (70.063-84.47425)	0.244	0.097	NS
	S2 T2	93.1435 (70.56137-97.743)	78.423 (56.94638-94.8545)	84.8815 (71.88438-92.22962)	0.533	0.043	NS
	S3 T1	75.9565 (65.837-92.25275)	70.846 (62.49325-83.3575)	86.21 (71.965-90.97725)	0.293	0.084	NS
	S3 T2	80.008 (73.78125-93.68812)	79.8205 (69.618-93.43863)	92.92525 (74.197-93.72825)	0.637	0.031	NS

Table 2: Values are presented as median (interquartile range, IQR). NS: Non-significant ($p>0.05$). The units of area measurement are in square micrometers (μm^2)**.** Statistically significant values ($p<0.05$) are shown in bold. The sample size for each group was 10. Group A: Fixed metal orthodontic appliance, Group B: Fixed ceramic orthodontic appliance, Group C: Removable orthodontic appliance. S: Site, T: Time-point

Table 3: Intragroup comparison of Group A across different sites and time -points

Parameter	N	Mean ± SD	Median (IQR)	Range	Wilcoxon W	P value	Effect size #
Cytoplasm Area S1							
T1	10	2386.247± 367.37202	2331.2587 (2240.9245,2685.4592)	1765.8855 to 2853.4725	10	0.084	-0.6364
T2	10	2406.4355± 425.69538	2437.224 (2069.6067,2754.4369)	1793.229 to 2916.2325			
Cytoplasm Area S2							
T1	10	2250.6409± 436.6928	2332.3655 (1855.136,2626.793)	1594.4445 to 2783.0725	27	1	-0.0182
T2	10	2141.2751± 326.78545	2175.217 (1931.7273,2327.8596)	1654.4385 to 2689.323			
Cytoplasm Area S3							
T1	10	2219.5112±367.78877	2126.684 (1946.722,2381.7444)	1803.3855 to 3010.25	19	0.432	-0.3091
T2	10	2169.5898±360.05731	2056.8573 (1983.2372,2196.6204)	1832.3855 to 3080.2955			
Micronuclei S1							
T1	10	7±1.76383	7 (5.25,8)	5 to 10	0.00	**0.008**	-1
T2	10	9.9±3.90014	9 (7.25,11.75)	5 to 17			
Micronuclei S2							
T1	10	6.3±1.63639	6.5 (5.25,7)	4 to 9	3.50	**0.028**	-0.8444
T2	10	8.8±3.01109	8 (7,10)	4 to 14			
Micronuclei S3							
T1	10	5.5±1.64992	6 (4,6.75)	3 to 8	5.5	**0.027**	-0.8
T2	10	7.6±2.71621	7 (5.5,9)	4 to 13			
N:C Ratio S1							
T1	10	0.0372±0.00843	0.0345 (0.0316,0.0438)	0.0268 to 0.0515	0	**0.002**	-1
T2	10	0.0417±0.01642	0.0356 (0.0331,0.0452)	0.0271 to 0.0826			
N:C Ratio S2							
T1	10	0.0337±0.00829	0.0323 (0.0288,0.0387)	0.0224 to 0.0461	0	**0.002**	-1
T2	10	0.0468±0.02532	0.0399 (0.0357,0.0449)	0.0292 to 0.1171			
N:C Ratio S3							

T1	10	0.0371±0.01089	0.0361 (0.0291,0.0458)	0.021 to 0.0527	0	**0.002**	-1
T2	10	0.0398±0.01033	0.0386 (0.0344,0.0465)	0.0212 to 0.0552			
Nuclear Area S1							
T1	10	86.7591±14.48649	80.9898 (77.7272,95.0738)	70.546 to 118.5765	9	0.064	-0.6727
T2	10	99.1406±41.9229	82.3105 (79.4921, 100.7897)	65.191 to 209.028			
Nuclear Area S2							
T1	10	74.2147±17.16377	70.6598 (60.3295,88.245)	52.985 to 98.9585	0	**0.002**	-1
T2	10	100.3405±57.3329	93.1435 (70.5614, 97.743)	57.7255 to 257.205			
Nuclear Area S3							
T1	10	79.7831±16.82806	75.9565 (65.837,92.2527)	61.85 to 111.4585	8	**0.049**	-0.7091
T2	10	84.1894±17.37952	80.008 (73.7813,93.6881)	65.2775 to 122.435			
# **Rank biserial correlation**							

Table 3: Group A (Metal fixed orthodontic appliance): Intragroup comparisons revealed no significant change in cytoplasmic area across sites ($p > 0.05$). Micronucleus frequency and N/C ratio showed significant increases at all sites ($p \leq 0.028$), with large effect sizes. Nuclear area showed significant increase at S2 and S3 ($p \leq 0.049$), with moderate-to-large effect sizes. The units of area measurement are in square micrometers (μm^2).

Table 4: Intragroup comparison of Group B across different sites and time -points

Parameter	N	Mean ± SD	Median (IQR)	Range	Wilcoxon W	P value	Effect size #
Cytoplasm Area S1							
T1	10	2321.2449±350.49565	2296.7585 (2140.9195, 2490.0098)	1750.8855 to 2840.535	15	0.232	-0.4545
T2	10	2137.22 ± 533.592	2137.2167 (2102.3101, 2328.3736)	1395.234 to 2879.76			
Cytoplasm Area S2							
T1	10	2202.3399±457.33123	2319.3645 (1829.385,2612.5408)	1528.194 to 2684.0725	30	0.846	0.0909
T2	10	2124.0729±314.11576	2151.717 (1936.9773,2334.5945)	1635.4385 to 2660.323			
Cytoplasm Area S3							
T1	10	2189.0992±381.27346	2131.649 (1953.472,2321.9944)	1708 to 3008.2	17.00	0.554	-0.2444
T2	10	2158.0898±357.84421	2042.3572 (1970.0698,2213.3704)	1824.3855 to 3080.2955			
Micronuclei S1							
T1	10	5.2±1.81353	5.5 (4.25,6)	2 to 8	0.00	**0.014**	-1
T2	10	8.5±3.68932	8.5 (6,10)	3 to 15			
Micronuclei S2							
T1	10	5±2.58199	4.5 (3.25,6)	2 to 10	6.00	0.055	-0.7333
T2	10	7.2±2.57337	6.5 (6,8.75)	3 to 11			
Micronuclei S3							
T1	10	4.4±2.54733	5 (2,5)	2 to 10	10.5	0.091	-0.6182
T2	10	6.4±2.71621	6.5 (4.5,7.75)	3 to 12			
N:C Ratio S1							
T1	10	0.036±0.00881	0.0337 (0.0301,0.0438)	0.02384 to 0.0501	27	1	-0.0182
T2	10	0.0372±0.0233	0.0342 (0.028,0.0418)	0.00624 to 0.0953			
N:C Ratio S2							
T1	10	0.0317±0.00846	0.0334 (0.0263,0.0376)	0.01785 to 0.0433	18	0.375	-0.3455
T2	10	0.0428±0.02627	0.035 (0.0285,0.043)	0.02722 to 0.115			
N:C Ratio S3							
T1	10	0.0362±0.01007	0.0357 (0.0288,0.0452)	0.0204 to 0.0511	7	**0.037**	-0.7455

T2	10	0.0393±0.0103	0.0377 (0.0341,0.0466)	0.02022 to 0.0541			
Nuclear Area S1							
T1	10	81.6535±15.18647	76.8485 (71.0941,87.1153)	67.5865 to 116.53	19	0.432	-0.3091
T2	10	98.1392±48.8072	79.8105 (71.1536,113.04)	58.99 to 222.028			
Nuclear Area S2							
T1	10	67.917±18.10308	63.312 (54.1936,83.745)	45.245 to 94.9585	9	0.064	-0.6727
T2	10	91.2404±57.65388	78.423 (56.9464,94.8545)	53.392 to 247.205			
Nuclear Area S3							
T1	10	75.1834±15.91627	70.846 (62.4933,83.3575)	59.85 to 109.4585	0	**0.002**	-1
T2	10	82.5853±18.21483	79.8205 (69.618,93.4386)	62.278 to 121.42			
# **Rank biserial correlation**							

Table 4: Group B (Ceramic fixed orthodontic appliance): Intragroup comparisons revealed no significant change in cytoplasmic area across sites ($p > 0.05$). Micronucleus frequency was significant only at S1 ($p = 0.014$), while N/C ratio and nuclear Area showed significance only at S3 ($p = 0.037$ and $p = 0.002$, respectively). The units of area measurement are in square micrometers (μm^2)**.**

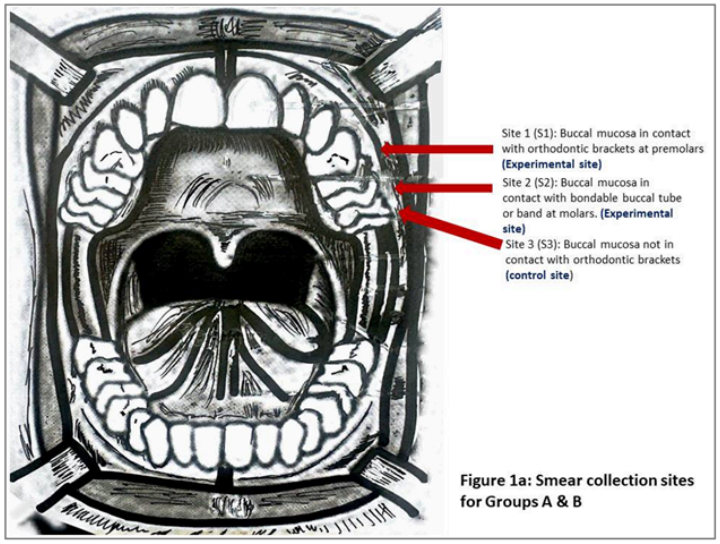
Table 5: Intragroup comparison of Group C across different sites and time -points

	N	Mean ± SD	Median (IQR)	Range	Wilcoxon W	P value	Effect size #
Cytoplasm Area S1							
T1	10	2089.2872±340.13464	1993.0813 (1815.5035, 2393.0833)	1706.18 to 2598.3965	10	0.084	-0.636
T2	10	2110.2645±347.98179	2017.2948 (1806.7928, 2412.9556)	1736.198 to 2609.6355			
Cytoplasm Area S2							
T1	10	2222.6896±414.38828	2333.4235 (1889.1212,2460. 2125)	1629.55 to 2816.253	0	**0.002**	-1
T2	10	2257.1631±394.06484	2363.3075 (1901.3136,2486.514)	1699.6525 to 2826.473			
Cytoplasm Area S3							
T1	10	2225.8094±379.36745	2209.8125 (1906.5217,2548.064)	1699.6525 to 2840.8855	0.00	**0.009**	-1
T2	10	2284.3023±388.65413	2357.7692 (1938.5201,2574.5225)	1699.6525 to 2880.7835			
Micronuclei S1							
T1	10	4.9±1.52388	4.5 (4,6)	3 to 7	5	**0.024**	-0.818
T2	10	6.9±2.76687	6 (5,9.25)	4 to 11			
Micronuclei S2							
T1	10	5.3±1.1595	5 (4.25,6)	4 to 7	0.00	**0.014**	-1
T2	10	8.3±2.49666	8.5 (7.25,9.75)	4 to 12			
Micronuclei S3							
T1	10	5.1±2.079	4.5 (4,6.75)	2 to 8	0	**0.006**	-1
T2	10	8.3±3.05687	8.5 (6.5,9.75)	3 to 13			
N:C Ratio							
S1 T1	10	0.0376±0.01006	0.0368 (0.0292,0.0432)	0.0255 to 0.0539	0	**0.002**	-1
T2	10	0.0402±0.00989	0.0389 (0.0325,0.0454)	0.0261 to 0.0557			
N:C Ratio S2							
T1	10	0.0371±0.01023	0.0376 (0.0273,0.0446)	0.0243 to 0.0513	0	**0.002**	-1
T2	10	0.0383±0.01097	0.0383 (0.0284,0.0451)	0.0246 to 0.0557			
N:C Ratio S3							
T1	10	0.0385±0.00752	0.039 (0.0344,0.0439)	0.0252 to 0.0482	0	**0.002**	-1
T2	10	0.04±0.00801	0.041 (0.0352,0.0473)	0.0262 to 0.0487			

Nuclear Area S1							
T1	10	76.8724±15.96999	77.332 (66.4936,87.8861)	53.819 to 103.295	0	**0.006**	-1
T2	10	82.6128±14.28696	81.3365 (76.7144,93.7715)	57.379 to 105.295			
Nuclear AreaS2							
T1	10	79.3114±13.55828	82.086 (70.063,84.4742)	60.351 to 104.781	0	**0.002**	-1
T2	10	83.0348±14.33202	84.8815 (71.8844,92.2296)	62.469 to 106.771			
Nuclear Area S3							
T1	10	84.4686±16.61367	86.21 (71.965,90.9772)	65.763 to 121.2675	0	**0.002**	-1
T2	10	82.5853±18.21483	79.8205 (69.618,93.4386)	62.278 to 121.42			
# **Rank biserial correlation**							

Table 5: Group C (removable appliance): Intragroup comparisons revealed significant changes in cytoplasmic area at S2 and S3 ($p \leq 0.009$) but not at S1 ($p = 0.084$). Micronucleus frequency, N/C ratio, and nuclear area showed statistically significant increases across all sites ($p \leq 0.024$), with strong to large effect sizes. The units of area measurement are in square micrometers (μm^2).

Figures



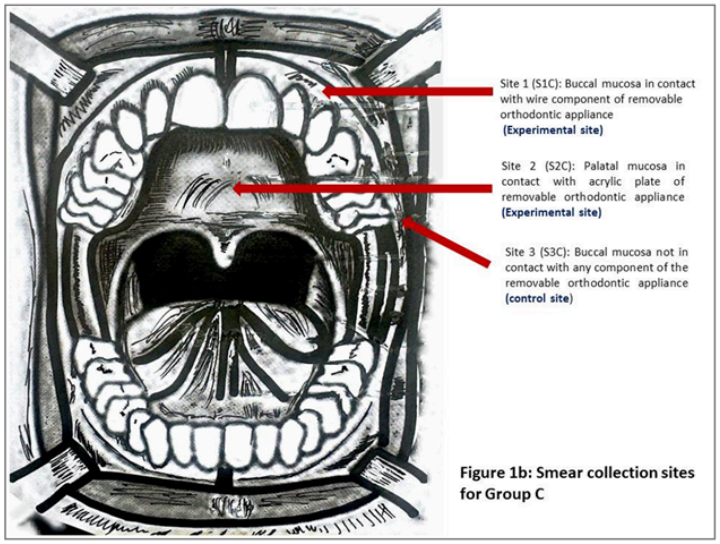


Figure 1

a: Intraoral sites for cytological smear collection in Groups A and B (fixed metal and ceramic orthodontic appliance groups)

Fig 1a**:** Figure depicting sites in Groups A and B where S1 and S2 represent experimental sites in direct or adjacent contact with metal and ceramic orthodontic appliance components, while S3 represents the control site with no direct appliance contact

b: Intraoral sites for cytological smear collection in Group C(Removable orthodontic appliance groups)

Fig 1b**:** Figure depicting sites in Group C where S1 and S2 represent experimental sites in direct or adjacent contact with removable orthodontic appliance components, while S3 represents the control site with no direct appliance contact.

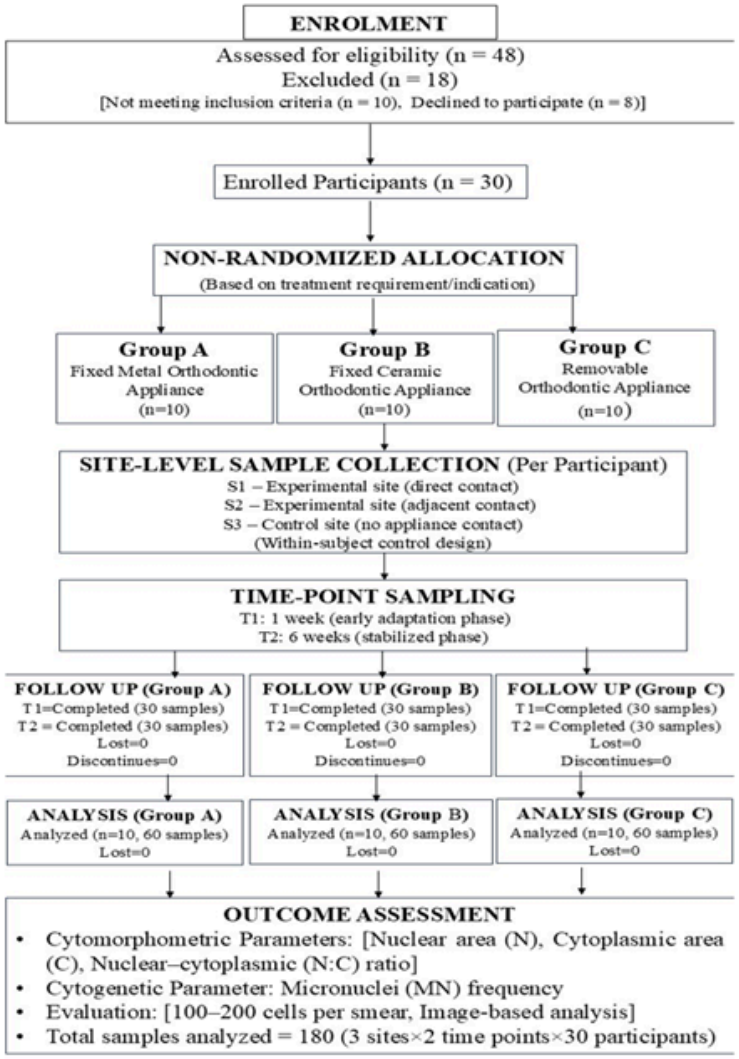


Figure 2

Participant flow diagram (CONSORT-adapted)

Fig 2: CONSORT style flow diagram adapted for non-randomized study. Of 48 participants screened, 30 were enrolled and allocated into three equal groups. Each participant contributed samples from three sites at two time points, yielding 180 samples analysed for cytomorphometric and cytogenetic parameters.



Figure 3a. GROUP A: Fixed Metal Orthodontic Appliance



Figure 3b. GROUP B: Fixed Ceramic Orthodontic Appliance



Figure 3c. GROUP C: Removable Orthodontic Appliance



Figure 3d. Papanicolaou staining kit used for cytological smear processing

Figure 3

Clinical orthodontic appliance groups and Papanicolaou staining (PAP) materials used in the study

Fig 3: Represents the clinical and staining material used. 3a: Fixed metal orthodontic appliance, 3b: Fixed ceramic orthodontic appliance, 3c: Removable orthodontic appliance, 3d: Papanicolaou staining kit used for cytological smear processing.

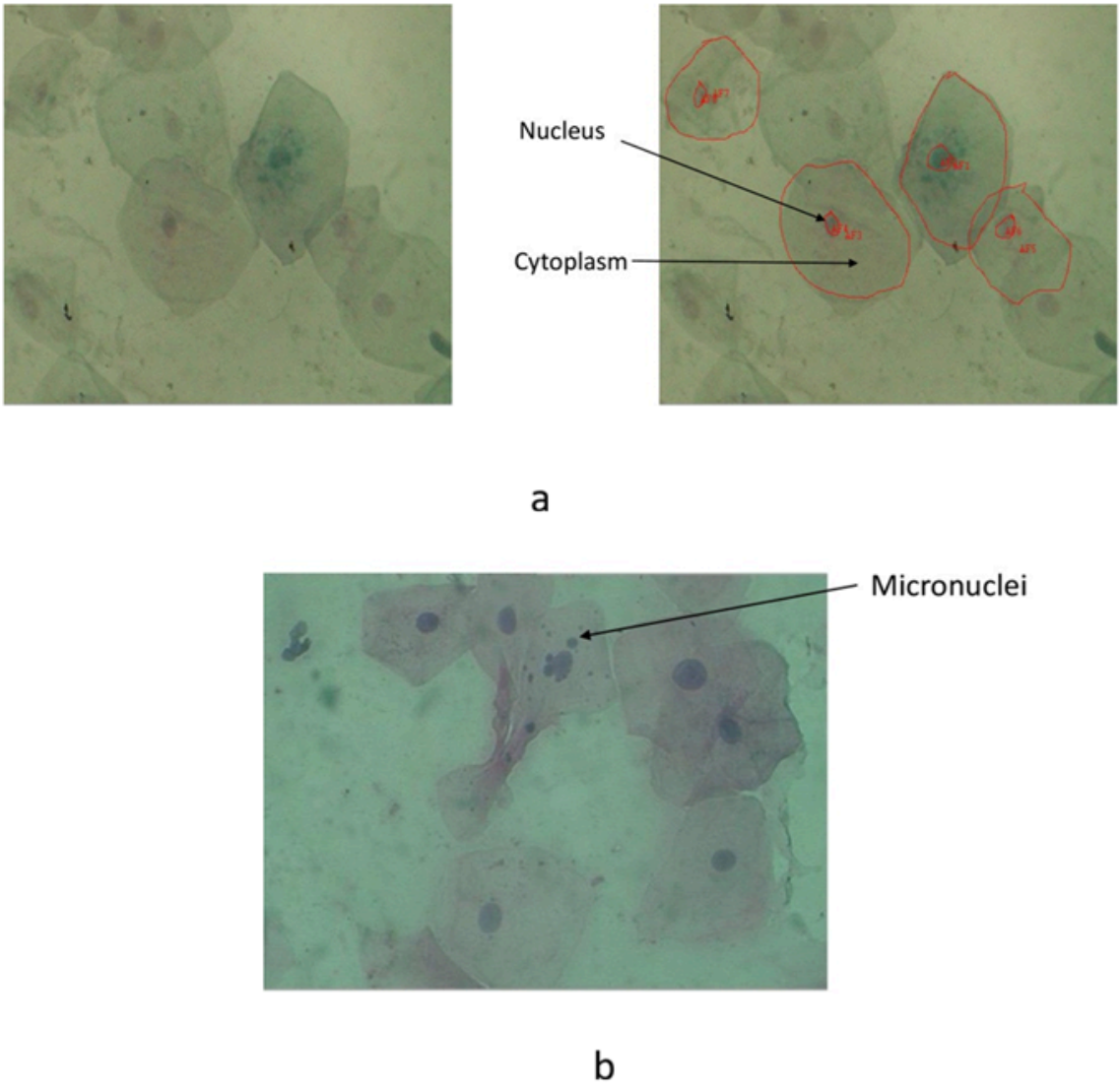


Figure 4

Representative Papanicolaou-stained smear demonstrating cytomorphology and micronuclei (MN) in exfoliated epithelial cells

a: Nuclear (N) and cytoplasmic (C) parameters marked with arrow. Area outline highlighted for cytomorphometric assessment (×40 magnification).

b: Micronuclei (MN) (arrow) are seen as distinct, rounded structures detached from the main nucleus and show similar staining characteristics.

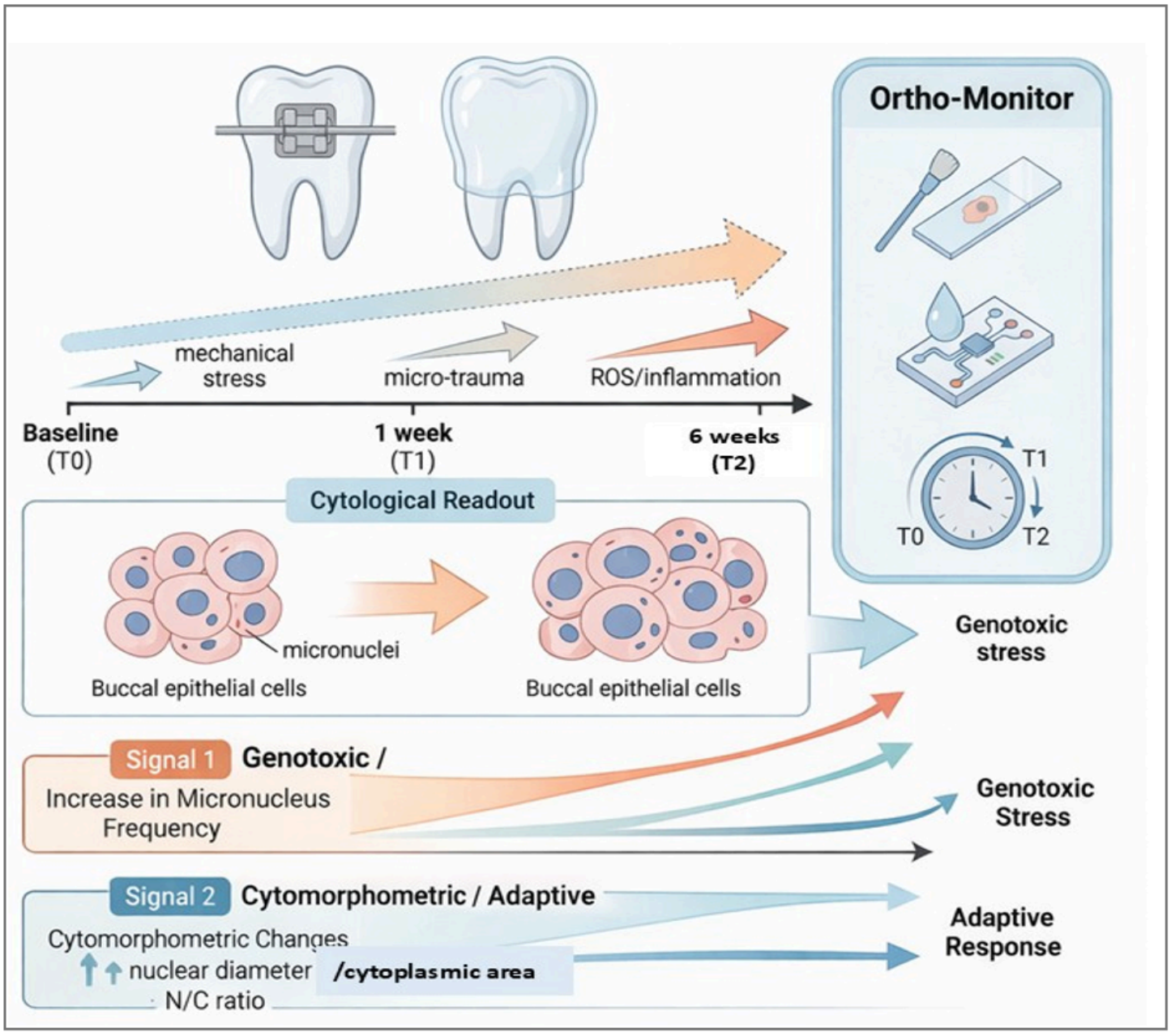


Figure 5

Conceptual Biomarker Model: Time-dependent Ortho-Monitor Framework

Fig 5: The model depicts a dual-signal pathway comprising (i) an early cytogenetic response (T1) reflected by increased frequency of micronuclei (MN), indicating cytological changes suggestive of genotoxic stress, (ii) a successive cytomorphometric adaptive response at T2 characterized by changes in nuclear area and nuclear–cytoplasmic (N:C) ratio