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Original Article

Cytotoxicity Assessment of the Effects of Pre-Orthodontic Trainer Appliances on Human Gingival Fibroblasts: An *in vitro* Study

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Main Points

- All 25 pre-orthodontic trainer appliances maintained cell viability above the ISO 10993-5 cytotoxicity threshold, indicating acceptable in vitro biocompatibility overall.
- Appliance-specific differences in cell viability were detected, although no statistically significant reductions were observed relative to the negative control.
- The K3 Blue (polyurethane/thermoplastic elastomer hybrid) appliance demonstrated the lowest cell viability and the most pronounced morphological alterations among the tested models.
- These findings emphasize the importance of appliance-level biocompatibility assessment, particularly for devices intended for prolonged intraoral use in children.

ABSTRACT

Objective: This study aimed to evaluate the *in vitro* cytotoxic effects of 25 pre-orthodontic trainer appliances on human gingival fibroblasts (HgnFs) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and morphological analysis.

Methods: Twenty-five pre-orthodontic trainer appliances were sterilized and incubated in Dulbecco's Modified Eagle Medium for 1 month to obtain conditioned eluates, in accordance with the principles of ISO 10993-5. The eluates were filtered and applied to cultured HgnFs for 48 h. Cell viability was assessed using the MTT assay, and morphological changes were evaluated using an inverted light microscope. Statistical analysis was performed at the appliance level using the Kruskal-Wallis test, followed by Dunn's post-hoc test ($p<0.05$).

Results: Statistically significant differences in cell viability were observed among the 25 appliance groups [Kruskal-Wallis H (25)=47.58, p=0.004]. The K3 Blue (A8) appliance demonstrated significantly lower viability than J2 (A12) and several other appliance models. Importantly, none of the tested appliances exhibited a statistically significant reduction in cell viability compared to the negative control group. All appliances maintained viability values above the ISO 10993-5 cytotoxicity threshold under the experimental conditions.

Conclusion: Within the limitations of this *in vitro* study, most pre-orthodontic trainer appliances demonstrated acceptable cytocompatibility with HgnFs. Appliance-specific differences were observed, highlighting the importance of individual appliance evaluation rather than broad material-based generalisations. Further studies incorporating long-term exposure models and in vivo validation are necessary.

Keywords: Pre-orthodontic trainer appliance, cytotoxicity, human gingival fibroblast, MTT assay, biocompatibility, myofunctional orthodontics

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INTRODUCTION

Craniofacial development and the aetiology of malocclusions are influenced by a complex interplay of genetic and environmental factors, including myofunctional habits. The significant impact of myofunctional habits on craniofacial development and malocclusion has been well documented for many years. There is a strong relationship between function and morphology, as the functions of chewing, speaking, breathing, and swallowing, together with the muscles of the jaw and face, directly influence dental, jaw, and facial development. Myofunctional treatment is an orthodontic method that aims to normalize impaired muscle and functional issues in the oral and jaw regions.[1]

The ideal breathing pathway is through the nose, which filters and humidifies air, ensuring better oxygenation when it reaches the lungs. For proper craniofacial development, the mouth should be closed, the teeth should be lightly in contact, and the tongue should rest against the palate. When the lip, cheek, and tongue muscles function correctly, jaw development is supported and the teeth can align physiologically. Mouth breathing, recognized since early years of orthodontics as a significant cause of malocclusion, leads to characteristic adaptations in affected individuals. Typically, the lips remain open, the tongue drops to a lower position, and abnormal swallowing patterns develop. These alterations disrupt the natural balance of forces on the teeth and jaws, resulting in various types of malocclusion classified as myofunctional disorders. Early treatment of these functional anomalies is crucial to prevent their progression into more severe morphological issues. The extent of craniofacial deformities due to habits depends on the nature of the movement, the magnitude of the force, and the duration and frequency of the habit.[2,3]

Pre-orthodontic trainer appliances are increasingly used for early intervention to address and correct harmful oral habits, guide favourable growth, and ensure adequate space for permanent teeth before more severe problems develop. The MYOBRACE® system (Myoresearch Co., Gold Coast, Queensland, Australia) is a widely utilized myofunctional appliance system designed to correct breathing disorders and myofunctional habits. These appliances are typically introduced in early childhood (aged 3-6 years) to promote desired craniofacial development, with usage protocols varying by appliance type, often including nightly wear and specified daytime hours of use.

The system comprises various series, including MYOBRACE® for Juniors (J1, J2, and J3), MYOBRACE® for Kids (K0, K1, K2, K3, K1 Broad, K2 Broad, and K3 Broad), MYOBRACE® for Teens (T1, T1 BWS, T2, T3, and T4), MYOBRACE® Interceptive Class III (i-3N, i-3, and i-3H), MYOBRACE® Permanent Dentition Class III (P-3N, P-3, and P-3H), and MYOBRACE® for Adults (A1, A2, and A3). Each series and model are designed with specific indications, functional features and usage protocols tailored to different age groups and malocclusion types.[4]

These appliances are typically manufactured from various polymeric materials such as silicone, polyurethane (PU), thermoplastic PU (TPU), and thermoplastic elastomers (TPE). Because these appliances remain in prolonged contact with oral tissues, their biocompatibility is paramount. Biomaterials are natural or synthetic substances used to perform or support the functions of living tissues in the human body. Due to their constant or intermittent contact with bodily fluids, these materials must possess specific properties such as mechanical strength, appropriate weight and density, wear resistance, suitability for mass production, aesthetic appeal, chemical stability, and fatigue resistance. Most importantly, biocompatibility, defined as the material's ability to interact with body tissues without causing harmful immunological or toxic reactions, is the primary criterion for patient safety, especially for orthodontic appliances intended for long-term use in the oral cavity. Silicone, for example, a polymer of silicon, is widely recognized for its high biocompatibility, flexibility and resistance to degradation.[5] Recent *in vitro* studies have specifically investigated the cytotoxic and biological effects of materials used in myofunctional and elastodontic appliances. Huang et al.[6] evaluated the biocompatibility and cytotoxic effects of myofunctional appliance materials on human periodontal ligament fibroblasts and reported material-dependent differences in cell viability and cellular morphology. Similarly, Dinu et al.[7] assessed the toxicological profile of elastodontic devices and demonstrated that material composition plays a critical role in the biological response of oral tissues. Despite their widespread use and long-term intraoral presence, limited literature exists on the cytotoxic effects of pre-orthodontic trainer appliances, particularly regarding the diverse range of materials and models currently available.

Therefore, the present study aimed to assess the *in vitro* cytotoxic effects of 25 pre-orthodontic trainer appliances on human gingival fibroblasts (HgnFs) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and morphological analysis in order to provide critical insights into their biocompatibility.

METHODS

This study was approved by the Biruni University Ethics Committee (approval number: BIAEK 2023/77-24, date: 06.01.2023).

Appliance Selection and Preparation

Twenty-five MYOBRACE® trainer models, representing various material categories including silicone, PU, TPU, TPE, and their combinations, were included in this study. No a priori power or sample size calculation was performed. The sample size was determined by the number of commercially available MYOBRACE® trainer models included in the system under investigation. Multiple technical replicates were used for each appliance to ensure experimental reliability. The specific models and their material compositions are detailed in Table 1.

Table 1. Myoresearch trainer appliances and their material compositions

Group	Model name	Material base
A1	A1	Silicone-based trainer
A2	i3-h	Polyurethane-based trainer
A3	A2	Silicone-based trainer
A4	P3	Polyurethane + thermoplastic elastomer-based
A5	T4B	Polyurethane-based trainer
A6	T4A	Polyurethane-based trainer
A7	i3-n	Silicone-based trainer
A8	K3 Blue	Polyurethane + thermoplastic elastomer-based
A9	T4K Soft Blue	Silicone-based trainer
A10	T4K Soft Pink	Thermoplastic elastomer-based trainer
A11	K1 Blue	Polyurethane-based trainer
A12	J2	Thermoplastic elastomer-based trainer
A13	K1 Clear	Polyurethane-based trainer
A14	T4A Hard Red	Silicone-based trainer
A15	TMD	Polyurethane-based trainer
A16	J1	Silicone-based trainer
A17	i3	Silicone + thermoplastic elastomer-based
A18	K3 Pink	Polyurethane + thermoplastic elastomer-based
A19	K2	Silicone + thermoplastic elastomer-based
A20	P3h	Polyurethane-based trainer
A21	Liptrainer	Silicone-based trainer
A22	K1 Pink	Silicone + thermoplastic elastomer-based
A23	Myochew	Silicone-based trainer
A24	Infant trainer	Silicone-based trainer
A25	T4A Clear	Polyurethane-based trainer

One representative material section was obtained from each trainer appliance. As some appliances consisted of multiple materials, the collected sections were intended to represent the combined material composition of each appliance, reflecting the clinical configuration rather than isolated materials. Sectioning was performed using a heavy-duty manual cutting instrument, and sample dimensions were standardized as far as possible (approximately 23×14 mm). No rotary instruments were used during sectioning to avoid heat generation or burr formation that could influence experimental outcomes.

All material sections were thoroughly cleaned under running water and subsequently sterilized using ultraviolet irradiation. After sterilization, the samples were handled under sterile conditions for eluate preparation.

Eluate Preparation

Each sterilized material section was placed in sterile 6-well culture plates. A fixed volume of Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic-anti-mycotic solution, was added to each well. The plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 1 month to allow the release of soluble components from the appliance materials.

This prolonged incubation period was selected to obtain eluates representative of cumulative material exposure over time, simulating long-term intraoral contact. Eluate preparation followed the general principles outlined in ISO 10993-5 and ISO 10993-12, without claiming full standardization of the surface area-to-volume ratio.

After incubation, the conditioned media (eluates) were collected and filtered through a 0.22-µm syringe filter to ensure sterility and remove particulate matter. Unconditioned DMEM served as the negative control.

Cell Culture

HgnFs were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained in a humidified incubator at 37 °C with 5% CO_2 and were harvested during the exponential growth phase for cytotoxicity experiments.

Cell Viability Assessment (MTT Assay)

HgnFs were seeded into 96-well microplates at a density of 2.0×10^4 cells/well and allowed to adhere for 24 h under standard culture conditions. After the attachment period, the culture medium was replaced with the prepared eluates or control medium, and the cells were exposed for 48 h.

Cell viability was assessed using the MTT assay. Following the exposure period, the eluates were removed and the cells were incubated with 100 µL of MTT solution (0.5 mg/mL in serum-free DMEM) for 4 h at 37 °C in the dark. The resulting formazan crystals were dissolved by adding 100 µL of dimethyl sulfoxide to each well. Absorbance was measured at 570 nm, with a reference wavelength of 630 nm, using a microplate reader. Cell viability was calculated as a percentage relative to the negative control group.

For each appliance-derived eluate, the MTT assay was performed using 12 technical replicates (wells) to minimize intra-assay variability. The mean absorbance of these technical replicates was calculated and used as a single data point representing that sample for statistical analysis.

Morphological Analysis

Following the 48-h exposure to the eluates, morphological changes in HgnFs were evaluated using an inverted light microscope (Nikon, Japan) at 20× magnification. Parameters such as cell detachment, membrane blebbing, cell shrinkage, and cytoplasmic granulation were qualitatively assessed and photographed.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, NY, USA). Data distribution was evaluated using the Shapiro-Wilk test. As the data did not follow a normal distribution, non-parametric statistical tests were applied. Differences among groups were analysed using the Kruskal-Wallis H test, followed by Dunn's post-hoc test with Bonferroni correction when appropriate.

The statistical unit of analysis was the appliance (n=25). Repeated MTT measurements were treated as technical replicates and not considered independent observations. A p-value of <0.05 was considered statistically significant.

RESULTS

The cytotoxic effects of the 25 Myoresearch trainer appliances on HgnFs were evaluated using the MTT assay and morphological analysis. Cell viability was calculated as a percentage relative to the untreated control group.

Cell Viability Assessment (MTT Assay)

The MTT assay results indicated statistically significant differences in cell viability among the various appliance groups [Kruskal-Wallis H (25)=47.58, p=0.004]. A post-hoc comparison with the negative control group demonstrated that none of the 25 appliance groups showed a statistically significant reduction in cell viability compared with the negative control group. This finding indicates that all tested appliances maintained cell viability above the ISO 10993-5 cytotoxicity threshold under these *in vitro* conditions.

However, statistically significant differences were observed when specific trainer-appliance groups were compared. For each appliance, a single viability value was obtained by averaging 12 technical replicates, and appliance-level values (n=25) were used for statistical analysis. Median values and interquartile ranges for each trainer group and for the control are presented in Table 2. The distribution of viability measurements across all groups is illustrated in Figure 1, showing medians, interquartile ranges, and outliers.

Overall viability trends: Most silicone-based trainer appliances, including A1, A3, A7, A9, A14, A16, A21, A23, and A24, demonstrated high cell viability, with most measurements exceeding 90% (e.g., A16 [J1] showed a median viability of 99.8%). These findings indicate a favourable *in vitro* biocompatibility profile for silicone-containing appliances.

Mixed-material groups containing silicone components, such as A17(i3), A19 (K2), and A22 (K1 Pink), also exhibited high cell viability, generally comparable to that observed in predominantly silicone-based appliances.

Polyurethane and thermoplastic elastomers-based appliances: Trainer appliances primarily composed of PU or TPE, or combinations thereof, demonstrated greater variability in cell viability results.

The K3 Blue (A8) group, composed of PU and thermoplastic elastomer, consistently exhibited the lowest cell viability among all tested appliances. Specifically, K3 Blue (A8) showed significantly lower viability compared with J2 (A12) ($p<0.05$) and with several silicone-based appliances (including A1, A3, A7, A9, A14, A16, A21, A23 and A24) (all $p<0.05$).

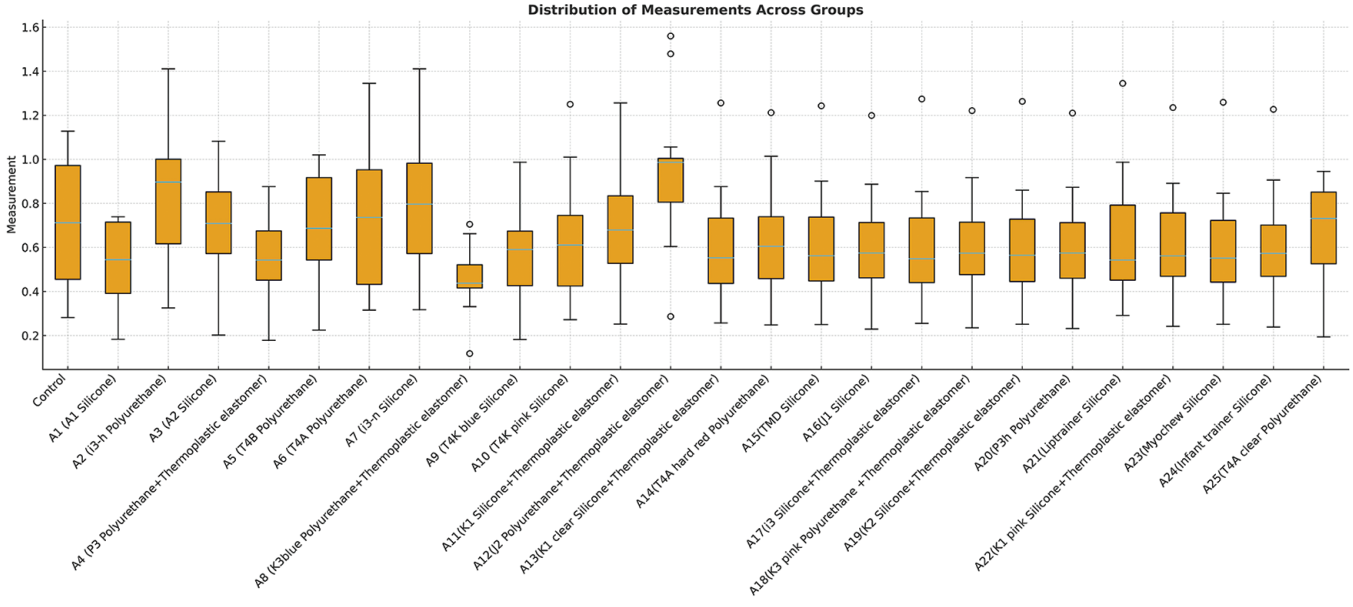

Figure 1. Box-and-whisker plots illustrating the distribution of cell viability values of human gingival fibroblasts following 48 h of exposure to 1-month-conditioned eluates obtained from 25 different myofunctional trainer appliances. Boxes represent the median and interquartile range,whiskers indicate minimum and maximum values, and dots represent outliers. Data are presented at the appliance level (n=25), with each value derived from the mean of 12 technical replicates.

Table 2. Median cell viability values (percentage of control) of human gingival fibroblasts (HgnFs) after 48 hours of exposure to 1-month-conditioned eluates obtained from 25 different myofunctional trainer appliances

Group	Model name & material base	Median absorbance (OD 570 nm)	Median cell viability (%) relative to control
Control	-	0.74	100.0
A1	A1 Silicone	0.51	68.9
A2	i3-h Polyurethane	0.89	120.3
A3	A2 Silicone	0.74	100.0
A4	P3 Polyurethane + thermoplastic elastomer	0.60	81.1
A5	T4B polyurethane	0.72	97.3
A6	T4A polyurethane	0.77	104.1
A7	i3-n Silicone	0.83	112.2
A8	K3 Blue polyurethane + thermoplastic elastomer	0.49	66.2
A9	T4K Soft Blue silicone	0.59	79.7
A10	T4K Soft Pink thermoplastic elastomer	0.66	89.2
A11	K1 Blue polyurethane	0.73	98.6
A12	J2 thermoplastic elastomer	0.99	133.8
A13	K1 Clear polyurethane	0.64	86.5
A14	T4A Hard Red silicone	0.67	90.5
A15	TMD polyurethane	0.65	87.8
A16	J1 Silicone	0.65	87.8
A17	i3 silicone + thermoplastic elastomer	0.64	86.5
A18	K3 Pink polyurethane + thermoplastic elastomer	0.65	87.8
A19	K2 Silicone + thermoplastic elastomer	0.65	87.8
A20	P3h polyurethane	0.65	87.8
A21	Liptrainer silicone	0.67	90.5
A22	K1 Pink silicone + thermoplastic elastomer	0.65	87.8
A23	Myochew silicone	0.64	86.5
A24	Infant Trainer silicone	0.65	87.8
A25	T4A Clear polyurethane	0.76	102.7

Each value represents one appliance (n=25) and was calculated as the mean of 12 technical replicates derived from the same eluate. Statistical comparisons were performed at the appliance level.

Other PU-based trainers, such as A2 (i3-h), A5 (T4B), A6 (T4A), A11 (K1 Blue), A13 (K1 Clear), A15 (TMD), A20 (P3h), and A25 (T4A Clear), generally demonstrated acceptable cell viability values. Although some of these appliances showed lower median viability values than silicone-based groups (e.g., approximately 85% for A6 and 88% for A20), these differences were not statistically significant compared with the control group.

Thermoplastic elastomer-based trainers, such as A10 (T4K Soft Pink) and A12 (J2), demonstrated acceptable cell viability, with A12 (J2) showing one of the highest viability values among the non-silicone groups.

Specific group comparisons: Statistical analysis confirmed that the viability of the K3 Blue (A8) group was significantly lower than that of multiple silicone-based appliances and selected TPE-based groups. In contrast, no statistically significant differences were observed among the silicone-based appliances themselves, indicating a consistently favourable biocompatibility profile within this subgroup.

Morphological Analysis

Microscopic examination of HgnFs following 48 h of exposure to 1-month-conditioned eluates provided qualitative findings that were consistent with the MTT assay results.

Control group: Cells exhibited normal spindle-shaped morphology, intact cell membranes, and confluent growth, indicative of healthy fibroblast proliferation.

Silicone-based groups: Cells exposed to eluates from silicone-based appliances generally maintained a healthy morphology, comparable to that observed in the control group. No marked morphological alterations, such as extensive cell detachment, membrane blebbing or pronounced cytoplasmic granulation, were observed.

Polyurethane and thermoplastic elastomer-based groups: Cells exposed to eluates from most PU- and TPE-based appliances exhibited minor to moderate morphological changes, including slight alterations in cell shape or reduced cell density; however, overall cellular integrity was preserved.

In contrast, cells exposed to eluates from the K3 Blue (A8) appliance displayed more pronounced morphological alterations. These changes included cell shrinkage, reduced substrate adherence, partial cell rounding, cytoplasmic granulation, and occasional membrane blebbing, indicating a stronger cellular response compared with other groups.

Overall, the morphological observations corroborated the quantitative MTT findings, demonstrating acceptable *in vitro* biocompatibility for all tested appliances and highlighting K3 Blue (A8) as the appliance associated with the most pronounced reduction in cell viability and morphological integrity.

DISCUSSION

The biocompatibility of materials used in pre-orthodontic trainer appliances is of critical importance because of their prolonged contact with oral soft tissues, particularly in growing children. This study evaluated the *in vitro* cytotoxic effects of 25 Myoresearch trainer appliances on HgnFs, providing insight into their biological response under controlled experimental conditions. Overall, the findings indicate that most of the evaluated appliances demonstrate acceptable cytocompatibility with HgnFs, which are consistent with their intended clinical use. The MTT assay, a widely accepted method for assessing cellular metabolic activity and viability, revealed that, although overall cytotoxicity was low, certain appliance models exhibited statistically significant differences in cytotoxicity.

A key observation in this study was that several silicone-containing trainer appliances (e.g., A3, A7, A9, A14, A16, A21, A23, and A24) demonstrated high cell viability and preserved cellular morphology, with values approaching those of the untreated control group. These findings are in agreement with previous reports describing the favourable biocompatibility profile of silicone-based materials in biomedical and dental applications.[8,9] Similarly, some mixed-material appliances incorporating silicone components (e.g., A17, A19, and A22) exhibited high viability, suggesting that the presence of silicone may contribute positively to the overall biological response of these appliances. Nevertheless, biocompatibility outcomes in the present study were evaluated at the appliance level, and the findings should not be interpreted as definitive evidence of the intrinsic superiority of any single material type.

In contrast, appliances primarily composed of PU- or PU-based hybrid materials exhibited variable cellular responses. Among these appliances, K3 Blue (A8) consistently demonstrated the lowest cell viability and more pronounced morphological alterations compared with several other models. This observation is consistent with previous studies reporting that certain thermoplastic and PU-based orthodontic materials may induce variable cytotoxic responses depending on their formulation and processing methods.[10-12] The reduced cell viability observed for this specific appliance may be associated with the release of residual monomers, oligomers, or degradation products from the polymer matrix, as well as manufacturing-related factors such as thermoforming or curing conditions.[13,14] Nevertheless, because the present study did not include chemical characterization of the eluates, no definitive conclusions can be drawn regarding the specific causative compounds. These findings are also consistent with recent *in vitro* studies evaluating the biocompatibility of myofunctional and elastodontic appliances. Huang et al.[6] reported variable cytotoxic responses among different myofunctional appliance materials when tested on human periodontal ligament fibroblasts, emphasizing that biological effects might differ substantially between appliance models despite similar clinical indications. Similarly, Dinu et al.[7] demonstrated that elastodontic devices exhibit material-dependent biological responses, highlighting the influence of polymer composition and manufacturing processes on cytotoxicity outcomes. In agreement with these studies, the present findings support the concept that appliance-specific characteristics, rather than broad material classifications alone, play a critical role in determining *in vitro* biocompatibility.

Despite the statistically significant differences observed among certain appliance models, most tested appliances did not show a significant reduction in cell viability compared with the control medium. This finding suggests that under the experimental conditions applied, the majority of pre-orthodontic trainer appliances exhibit acceptable short-term *in vitro* cytocompatibility. However, the detection of reduced viability and morphological alterations in specific models highlights the importance of appliance-specific evaluation rather than broad, material-based generalizations, particularly when these devices are intended children with developing oral tissues.

Myofunctional trainer appliances, including the MYOBRACE® system evaluated in this study, play an important role in early orthodontic intervention.[15] Their clinical effectiveness depends not only on their functional and mechanical properties but also on their biological safety during prolonged intraoral contact.[16] While the present *in vitro* findings provide valuable preliminary information, the oral environment is highly dynamic, and factors such as salivary flow, enzymatic activity, pH fluctuations, temperature changes and mechanical loading may influence material behaviour and biological responses *in vivo*.[17]

From a materials science perspective, the relatively favourable cellular response observed in silicone-based appliances may reflect the principle that materials exhibiting high chemical stability and resistance to degradation tend to elicit fewer adverse biological reactions. In the context of long-term tissue contact, biocompatibility has traditionally been linked

to the concept of chemical and biological inertness and the minimization of degradation products that may elicit inflammatory or cytotoxic responses.[13,14] Silicone polymers have historically been grouped among materials that are relatively resistant to degradation and are used in implantable medical devices. These considerations may partly explain the higher cell viability observed in silicone-containing appliances in this study, although biocompatibility remains dependent on specific formulations and processing characteristics.

Study Limitations

Several limitations of this study should be acknowledged. As an *in vitro* investigation, the experimental conditions cannot fully replicate the complex and dynamic environment of the oral cavity. Additionally, the study focused on overall cell viability and morphological changes, and did not include chemical analysis of eluates, which would be necessary to identify specific compounds responsible for the observed biological effects. Cytotoxicity was assessed following a 48-h exposure period, and longer-term or repeated exposures may yield different cellular responses. Furthermore, only HgnFs were evaluated; inclusion of additional oral cell types or in vivo studies would provide a more comprehensive understanding of biological safety.

CONCLUSION

Within the limitations of this *in vitro* study, most evaluated MYOBRACE® trainer appliances demonstrated acceptable cytocompatibility with HgnFs. However, certain appliance models incorporating PU-based hybrid materials, particularly K3 Blue (A8), exhibited reduced cell viability and discernible morphological changes.

These findings emphasize the importance of appliance-specific biocompatibility assessment and suggest that material composition and manufacturing characteristics should be carefully considered when selecting myofunctional trainer appliances for clinical use, especially in paediatric patients.

Further studies incorporating chemical characterization of eluates, long-term exposure models, additional cell types, and in vivo validation are warranted to better elucidate the biological behaviour and clinical safety of these widely used orthodontic devices.

Ethics

Ethics Committee Approval: This study was approved by the Biruni University Ethics Committee (approval number: BIAEK 2023/77-24, date: 06.01.2023).

Informed Consent: Not applicable (*in vitro* study).

Footnotes

Author Contributions: Concept - Z.M.T., M.G.; Design - Z.M.T., M.G.; Data Collection and/or Processing - Z.M.T.; Analysis and/or Interpretation - Z.M.T., H.G.G., M.G.; Literature Search - H.G.G.; Writing - Z.M.T., H.G.G.

Conflict of Interest: No conflict of interest was declared by the authors.

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