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Original Research Article

Effects of commercially available clear aligner plastics on human gingival mesenchymal stem cells

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Abstract

Background: Recent innovations in clear aligner materials have resulted in enhanced flexibility and resistance to staining through multilayer and multiphasic designs. Integrating digital technologies such as intraoral scanning, tooth movement planning software, and 3D printing has significantly streamlined the workflow for clear aligners. These aligners are designed to be worn for over 20 hours daily, necessitating high biocompatibility from the materials used. While these materials undergo rigorous cytotoxicity testing, there are conflicting findings regarding their cytotoxic assessment.

Materials and Methods: This in vitro study investigated the cytotoxic effects of four different clear aligner materials - TAC-Polyethylene terephthalate glycol (PET-G), Flash-Polyethylene terephthalate (PET), Taglus Premium-Polyethylene terephthalate (PET), and Invisalign-SmartTrack (multi-layer aromatic thermoplastic polyurethane) - on gingival mesenchymal stem cells. The preformed clear aligners were applied in a powdered form to the cells, which were subsequently harvested at three time points (TP1, TP2, and TP3) and assessed for cytotoxicity using the MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide].

Results: Colorimetric MTT assay was used to estimate the number of active and viable cells. Every sample was tested at three different time frames against the control. The most amount of cell growth was seen on TAC aligner material with P = 0.06776 followed by Invisalign (P = 0.23559), Flash (P = 0.35465), and Taglus (P = 0.52129). However, none was statistically significant. In

Conclusion: All materials for clear aligners showed no cytotoxicity under the experimental conditions.

Keywords: Orthodontics, Clear aligners, Clear aligner cytotoxicity, MTT assay, Invitro cytotoxicity, Bisphenol A, Bisphenol A release, BPA toxicity, Invisalign, TAC, Flash, Taglus, Mesenchymal cells, mesenchymal stem cells, stem cells, human, gingival cells, gingival fibroblasts, human gingival mesenchymal stem cells, invitro

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1. Introduction

Clear aligners have revolutionized the field of orthodontics, offering a transformative approach to tooth movement and alignment. Aesthetics, tooth movement, removability, and customization are key aspects of clear aligner therapy, providing patients with a discreet and efficient treatment option. Recent advances in material science have led to the development of multilayer and multiphasic properties, enhancing flexibility and stain resistance. The integration of digital technologies, including intraoral scanning, software for tooth movement planning, and 3D printing, has streamlined the clear aligner workflow.[1] Furthermore, in-office production capabilities have expanded treatment options, making clear aligner therapy an essential component of contemporary orthodontics. The growing preference for aesthetic orthodontic interventions extends beyond adults to encompass adolescents and children[2], thereby increasing the prevalence of clear aligner therapies.

These materials fit snugly around the teeth and adjacent gingiva, predominantly to the marginal one-third region of the gingiva.[1] Aligners are replaced every two weeks, with each set typically worn for 22 hours daily for 10 to 14 days throughout the entire treatment course.2 The duration of treatment generally ranges from 6 months to 3 years,

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contingent upon the severity of the individual case.[3] Throughout this period, the aligners maintain constant contact with intraoral structures such as teeth, gingiva, and oral fluids.

Orthodontic aligner appliances utilize a variety of materials to achieve optimal performance. These materials include Polyethylene terephthalateco-1, 4 cylclohexylenedimethylene terephthalate (PETG), thermoplastic polyurethane (TPU), and copolyester polyethylene terephthalate (PET).4 PETG is valued for its robustness and flexibility, making it suitable for forming the structural base of aligners. TPU, known for its elasticity and durability, contributes to the aligners' ability to apply gentle yet effective forces for tooth movement. Copolyester PET offers a balance of strength and clarity, ensuring aligners are both durable and aesthetically pleasing.

Nevertheless, there are concerns about the possible leaching of bisphenol A (BPA) which may arise from these biomaterials, resulting in negative outcomes. Research has shown that these plastic materials can show cytotoxicity and result in biological responses, such as altered gene expression, immune responses to material exposure, disruption of the cell cycle, apoptosis, and the induction of mutagenesis or carcinogenesis.[2-3] However, conflicting findings have emerged, as certain studies indicate the absence of cytotoxic effects on oral epithelial cells while noting changes in their pattern and the expression of proteins related to inflammatory responses.[4] The proliferation of new aligner software companies in recent years highlights the critical need to evaluate the cytotoxicity of materials utilized across different brands.[1]

During the wear period, clear aligners undergo mechanical, physical, and thermal stresses.[5] As they tightly conform to the gingiva, leached byproducts from the aligners can promptly interact with gingival cells, potentially compromising their integrity. Therefore, assessing the cell-to-cell barrier function and the permeability of cells exposed to aligner materials is crucial.

Cell culture methods provide a straight forward, reproducible, cost-effective, and meticulously controlled approach to assess the cytotoxic effects of dental materials.[1,6] Previous studies have investigated the impact of clear aligners on gingival stem cells using either the filtrate from clear aligners or particles of the non-thermoformed material to assess cytotoxicity.**Error! Reference source not found.**

The use of aligner filtrate is limited by the reduced release of leached products, making it non-standardized. Moreover, thermoforming alters the properties of aligner materials, potentially leading to the release of substances such as Bisphenol A.[7-11] Hence, there is a need to standardize the testing by using thermoformed aligners for cytotoxicity.

Therefore, this study aims to assess the in vitro cytotoxicity of various thermoplastic materials that are used for clear aligners on human gingival stem cells. The null hypothesis posited no variance in cytotoxicity among the different materials tested.

2. Materials and Methods

This in vitro study investigated the cytotoxic effects of four different clear aligner materials - TAC-Polyethylene terephthalate glycol (PET-G), Flash-Polyethylene terephthalate (PET), Taglus Premium-Polyethylene terephthalate (PET), and Invisalign-Smart Track (multi-layer aromatic thermoplastic polyurethane PU) - on gingival mesenchymal stem cells. The preformed clear aligners were applied in a powdered form to the cells, which were subsequently harvested at three time points (TP1, TP2, and TP3) and assessed for cytotoxicity using the MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide].

The Scientific and Ethics research committee at the University approved this prospective study. The calculation of the sample size was done using G-Power software 3.1.9.7 with convenience sampling and was based on a significance level of 95%.

The inclusion criteria were, healthy gingival mesenchymal stem cells obtained from the Stem Cell Regeneration Laboratory of the university and unused, fresh, readymade, prefabricated clear aligners (Invisalign, Illusion, Tac, Flash clear aligners) Cells from inflamed gingival tissue and used aligners were excluded from the study.

2.1. Materials, cell preparation:

The cell preparation was carried out by the university's Stem cell and Regenerative Laboratory. Primary Human Gingival Fibroblasts (HGF) are procured from healthy patients of the age group between 20-30 years.

Tissue fragments are rinsed twice in phosphate-buffered saline solution and cut into 1mm size. These fragments are placed on the tissue culture dishes. These dishes are then placed in an incubator with humidification of 5% CO2, in Dulbecco's Modified Eagle Medium (DMEM) augmented with 10% Fetal Bovine Serum, 2 mM glutamine, 100 U/mL of penicillin, and 100 lg/mL of streptomycin, all of which were at 37**°**C.11,12 Fibroblasts started proliferating after 10 days. Once a substantial cellular network was obtained, the cells were treated with phosphate-buffered solution and removed from the culture dishes. This involved treating with trypsin/ethylenediaminetetraacetic acid for 5 minutes followed by re-culturing until a dense single layer was regenerated.

2.2. Sample preparation

Four materials are assessed, Invisalign, Illusion, Tac, Taglus premium.

Pre-made aligner sets are used, and the plastic is ground into coarse particles using a tungsten carbide bur no. 699, 0.9 mm in diameter.

Before culture, 70% alcohol is used for washing and UV sterilizing. Three samples of each clear aligner material are used.

Table 1: Materials used in the study.

Aligner	Material	Manufacture
TAC	Polyethylene terephthalate glycol (PET-G)	The Aligner Company, India
Flash	Polyethylene terephthalate (PET)	3D Future Technologies Pvt. Ltd. India
Taglus Premium	Polyethylene terephthalate (PET)	TAGLUS, India
Invisalign	SmartTrack (multi-layer aromatic thermoplastic polyurethane PU)	Align Technology Santa Clara, CA, United States

Table 1: Materials used in the study

2.3. Cell viability and vitality assessment

MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] is carried out to test cell viability. Human gingival fibroblasts are seeded into 96-well flat-bottomed tissue culture plates at a density of 10,000 cells per well.

Following a 24-hour incubation period, 200 lL/well of the extract was added to the culture media. The media was changed to 100 lL/well of MTT solution (1 mg/mL) in PBS after a further 24 hours, and the cells are then incubated for an additional hour at 37 degrees Celcius in an environment containing 5% CO2. Following the removal of the solution, 100 lL of dimethyl sulfoxide per well is mixed, and stirred for about 7-10 minutes.

Each well's optical density can be determined at 590 nm using a spectrophotometer (*Sunrise, Team, Mannederf, Zurich, Switzerland*). Para rubber is used as a positive control, and the optical density of the cells grown in the DMEM media in the absence of any transparent aligner material is measured. Sample extracts are utilized as a reference point to ascertain the assay's level of cytotoxicity as well as a control for 100% cell viability. Separate experiments are carried out in triplicate.

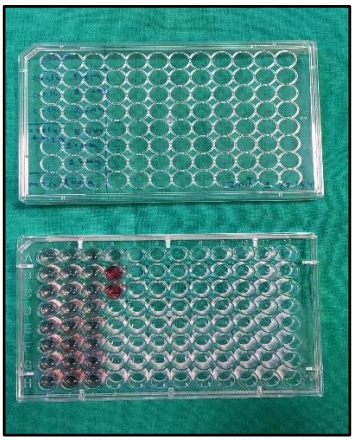

Figure 1: MTT assay carried out

2.4. Statistical analysis

The Statistical Package for Social Sciences was used to conduct statistical analysis and descriptive statistics (SPSS 22.0, SPSS IBM, Armonk, NY, USA). The normal distribution of data was verified by the Shapiro-Wilk test.

The Bonferroni post-hoc test was used in both one-way and two-way analysis of variance (ANOVA) to assess differences between mean values.

A significant threshold of $P \leq 0.05$ was established.

3. Results

The estimation of the number of active and viable cells is done by colorimetric assay. Every sample is tested at three different time frames against the control.

The average value of each along with the standard deviation is given in Table no. 2. The four materials along with the control are checked at three different time frames (T1, T2, T3). For the control, cell proliferation can be seen increasing successively at each time frame from T1 to T3. This value is compared with the four materials for cell viability. An increase in the number of cells on the material surface indicates zero cytotoxicity of the material.

Table 2: Absorbance values of the materials used.

Materials	T1	T2	T3	Average	Std	P value
Control	0.1294	0.2090	0.3485	0.1717		
TAC	0.2874	0.4045	0.1820	0.2913	0.1113	0.06776
Taglus	0.1181	0.7413	0.1859	0.3484	0.34192	0.52129
Invisalign	0.5517	0.1960	0.2238	0.3238	0.19783	0.23559
Flash	0.2024	0.8080	0.1956	0.4020	0.35162	0.35465

The bar (**Figure 1** represents the absorbance value in nanometres (Y axis) and the material used (X-axis). (x axis – material; y axis – absorbance 560nm)

A comparison of the test sample with the control can be done to analyse the toxicity of the said material. All the materials showed no cytotoxicity to the gingival stem cells and all of them had cell proliferation on their surfaces.

The most amount of cell growth is seen on TAC aligner material with P = 0.06776 followed by Invisalign (P = 0.23559), Flash (P = 0.35465), and Taglus (P = 0.52129). However, none is statistically significant.

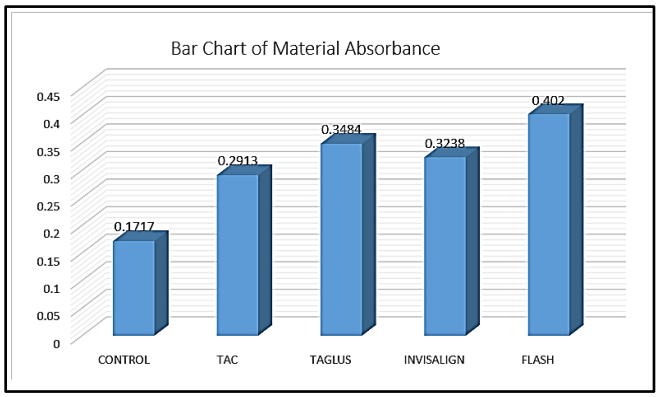

Figure 2: Material absorbance

4. Discussion

Recent advancements in biomaterials alongside the integration of computer-aided design (CAD) and manufacturing (CAM) technologies have propelled clear aligner therapy (CAT) to be a viable substitute for conventional fixed brackets in orthodontic practice.[12] Over the past decade, there has been a notable surge in demand for CAT, largely attributed to robust marketing campaigns by commercial clear aligner firms employing direct-to-consumer advertising and leveraging social media platforms.[13] This widespread promotion has significantly elevated public awareness regarding aesthetic alternatives in orthodontic treatment, particularly among adult patients.[14-15]

Currently, aligners are manufactured using two main methods: traditional vacuum thermoforming with thermoplastic materials on physical models and, direct 3D printing without the need for intermediate physical models.[16] The vacuum thermoforming process is widely utilized in both commercial production and clinical settings, including in-house aligner fabrication.[3,17] However, aside from Tera Harz TC-85 (Graphy, Seoul, South Korea), which has obtained approvals from the Korea Food and Drug Administration (KFDA), European Commission (EC), and Food and Drug Administration (FDA) according to the company's website, there are currently no other commercially available 3D printable materials that meet the required standards of biocompatibility, translucency, and appropriate mechanical properties.[18]

The 3D-printed plastics are manufactured by either computer-aided design or computer-aided manufacturing technology (CAD/CAM).[19-20] These technologies work through additive methods (successive layering), subtractive methods (grinding or milling), or liquid materials (stereolithography). The raw material for these procedures includes cytotoxic materials like Polymethyl methacrylate (PMMA). When examining the manufacturing of clear aligners using the thermoforming process, it becomes evident that this method can result in surface irregularities that may complicate the treatment.[21] The thermoforming procedure, characterized by rigorous heating and curing cycles, raises concerns about potential toxicity to the oral cavity.[22] Efforts are made to reduce the cytotoxicity of such materials by curing, polishing, or sterilization including autoclaving or gamma radiations.[23] However, these findings are not consensual. Moreover, mechanical strength is reduced because of these procedures.

The process of conversion of monomers into polymers can lead to incomplete removal of monomers. A significant reduction in the degree of conversion can also increase the release of monomers such as methyl methacrylate (MMA), triethylene glycol dimethacrylate (TEGDMA), 2-hydroxyethyl methacrylate (HEMA), and bisphenol A glycidyl methacrylate (Bis-GMA).20[-24] These monomers have the potential to cause both systemic and local adverse effects, including teratogenicity and estrogenicity, as well as cytotoxicity and mutagenicity.[22-24] The breakdown and metabolism of these monomers have the potential to permanently harm cellular DNA. Other authors have shown that oxidative stress is elevated and glutathione sequestration is induced.[25-26] These occurrences have the potential to alter the cell cycle and ultimately cause apoptosis-induced cell death.

Despite their ISO certification for biocompatibility or marketing claims, Rogers et al.[27] observed significant reproductive damage in murine oocytes when exposed to the ingredients used in the fabrication of clear aligners. Regretfully, these results aren't often apparent right away. Conversely, Eliades et al. soaked aligners (Invisalign) in a saline solution for two months at 37**°** Celsius in a glass container before discovering no cytotoxicity from the aligners.[2] However, it is important to highlight that the material described in the study by Eliades et al.[2] was subsequently replaced with SmartTrack material, which underwent testing in 2012.

It was concluded that Invisalign material had traces of bisphenol A, which were insufficient for leaching out. According to Premaraj, isocyanate, another ingredient in the Invisalign material, may have an impact on oral health and cause allergic reactions.

In our study, we have compared four different clear aligner materials for cytotoxicity. Gingival mesenchymal stem cells (GMScs) were chosen to evaluate the cytotoxicity

because, along with epithelial keratinocytes, they represent the predominant cell type in oral tissues and are directly exposed to potential harm from thermoplastic materials, as aligners come into contact with the gingiva. When evaluating dental materials in vitro, the International Standards Organization (ISO) advises utilizing Human Gingival Fibroblasts (HGFs) since these cells are frequently used to assess the biocompatibility of dental materials.28 On day 14, under the experimental conditions, all the materials exhibited good cell viability levels and no cytotoxic effect. The toxicity levels observed with various other dental materials, such as metallic brackets and bands, mini-screws, and bonding materials.[1,3,4,6]

In vitro cytotoxicity was examined in this investigation employing gingival fibroblasts. The authors of the current investigation found that in a saline solution environment, exposure to the plastic did not affect the adhesion, membrane permeability, or survival of epithelial cells. There was proliferation of cells on the material suggesting that the plastics were completely nontoxic. In vivo studies can also suggest the role of saliva postulating that saliva might offer additional protection. The results of this study proved the null hypothesis that there is no cytotoxicity by the aligner materials. This could be because the release of BPA is increased in an alkaline environment.

It is important to acknowledge that in vitro methods are not able to fully replicate the intraoral environment. Intraoral insults affect the properties of thermoplastic materials of aligners, which could also affect biocompatibility.

The aligner is exposed to consistent and occasional forces linked to routine oral activities such as chewing, speaking, and swallowing, as well as parafunctional habits like teeth clenching and grinding. Microcracks, delamination, calcified biofilm deposits, and reduced transparency have been noted in Invisalign aligners used over a two-weeks.28 Intraoral hygroscopic expansion can alter the aligner's fit and modify the orthodontic forces it applies. Thermoplastic materials, particularly those used in Invisalign aligners and PETG, show increased water absorption over time.29 Following consumption of a hot beverage, the temperature in the oral cavity can rise to 57°C and may take several minutes to return to normal. These temperature fluctuations have been shown in various in vivo and in vitro studies.30 to affect the mechanical properties of thermoplastic materials. The wear and tear experienced by aligners could potentially affect their leaching properties, highlighting the necessity for further research in this area.

5. Conclusions

In summary, all materials used for clear aligners demonstrated non-cytotoxicity under the experimental conditions. Since materials for clear aligners have no clinical as well as statistically significant cytotoxicity, their clinical use should be considered safe.

6. Source of Funding

None.

7. Conflict of Interest

None.

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