Journal of Dental Sciences 19 (2024) 2065–2073

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jds.com

Original Article

The cytotoxicity assessment of different clear aligner materials

I-Lin Lo [a†], Chuan-Yi Kao [b,c†], Tsui-Hsein Huang [a,d], Chun-Te Ho [a,d], Chia-Tze Kao [a,d*]

[a] School of Dentistry, Chung Shan Medical University, Taichung, Taiwan
[b] Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan
[c] Department of Psychiatry, Chung Shan Medical University Hospital, Taichung, Taiwan
[d] Department of Dentistry, Chung Shan Medical University Hospital, Taichung, Taiwan

Received 9 May 2024; Final revision received 20 May 2024
Available online 31 May 2024

KEYWORDS
Aligner;
Thermoformed;
Periodontal ligament cell;
Viability

Abstract *Background/purpose:* Invisible orthodontic treatments are becoming increasingly popular, and numerous brands of invisible aligners are now available. However, concerns remain about the safety of the materials used in these products. This study aimed to assess the cytotoxic effects of both original and thermoformed thermoplastic materials used in orthodontic aligners on human periodontal ligament (HPDL) cells in vitro.
Materials and methods: The experiment used six different brands, each containing three types of thermoplastic materials, Polyethylene terephthalateco-1, 4-cyclohexylenedimethylene terephthalate (PETG), thermoplastic polyurethane (TPU), and copolyester polyethylene terephthalate (PET). The original sheets and the thermoformed materials were soaked in a culture medium for seven and fourteen days, and then applied to cultured human periodontal ligament cells. Cells were harvested on the first, third, and fifth days after application, and their viability was analyzed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay.
Results: The findings revealed that some thermoformed materials, notably PETG, exhibited lower survival rates compared to their non-thermoformed versions. However, other materials such as TP and PET maintained over 70% cell viability, indicating only minor cytotoxic effects.
Conclusion: These findings highlight the need for further research into the long-term biocompatibility of these materials but generally affirm their safety for use in orthodontic aligners under the tested conditions.

* Corresponding author. School of Dentistry, College of Oral Medicine, Chung Shan Medical University, Department of Orthodontics, Chung Shan Medical University Hospital, No. 110, Chien Kuo N. Road, Taichung, 00407, Taiwan.
E-mail address: ctk@csmu.edu.tw (C.-T. Kao).
† These two authors had equal contribution to this study.

https://doi.org/10.1016/j.jds.2024.05.025

Introduction

The modern orthodontic aligner appliance, now widely embraced by patients, undergoes a streamlined fabrication process. It begins with the digitization of oral scans to create a three-dimensional model of the dentition. This model serves as the foundation for crafting the final orthodontic aligner through precision molding with thermoplastic plates.

Various materials are employed in orthodontic aligner appliances, including Polyethylene terephthalateco-1, 4-cylclohexylenedimethylene terephthalate (PETG), thermoplastic polyurethane (TPU), and copolyester polyethylene terephthalate (PET). However, concerns have been raised regarding the potential release of bisphenol A (BPA) from these biomaterials, which could lead to adverse effects. Studies have indicated that these plastic-based materials may exhibit cytotoxicity and induce biological reactions, including modified gene expression.[1,2] Nonetheless, conflicting findings exist, with some studies suggesting no cytotoxic effects on oral epithelial cells but alterations in their behavior and the expression of inflammatory response-related proteins.[3] While the majority of BPA release originates from the aligner material, there appears to be no significant discrepancy in BPA concentration among different types of aligners across various media.[4]

Due to the intricate nature of the oral environment, factors like acidic beverages, enzymatic activity, mastication, and attrition force can contribute to the abrasion and wear of clear aligners, leading to increased particle release. A study demonstrated slight cytotoxicity associated with this phenomenon, with PETG showing the highest cytotoxicity, particularly exacerbated by the thermoforming process.[5] A systematic review underscored the potential safety concerns surrounding aligner appliances, highlighting the necessity for further clinical investigations into their biocompatibility.

Therefore, the objective of this study was to examine the in vitro cytotoxic effects of various thermoplastic materials used for clear aligners on human periodontal ligament cells (HPDLs).

Materials and methods

Cell culture

The HPDLs was bought from ScienCell Research Laboratories Cat. #2630 (ScienCell Research Laboratories, Carlsbad, CA, USA).To cultivate human periodontal ligament cells, begin by preparing Dulbecco's Modified Eagle Medium (DMEM, Gibco, ThermoFisher Inc, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, ThermoFisher Inc, Waltham, MA, USA) and 1% penicillin-streptomycin, at a dosage of 100 units per ml of DMEM. Introduce the cells to this medium and incubate at 37 °C in a CO_2 incubator set to 5% CO_2 for three days. Once the cells adhere to the bottom of the culture dish, indicating successful formation, transfer them to a similar 37 °C, 5% CO_2 incubator for further passaging.

Thermoplastic plate preparation

Thermoplastic plate preparation: Totally three types (PETG, TPU, PET), six brands of thermoplastic plates divided into two groups: original and thermoformed, are shown in Table 1. Thermoforming is done using the ESSIX® Select Vac® vacuum thermoforming machine (Ortho Technology, Inc. West Columbia, SC, USA), according to the heat treatment seconds specified by each brands.

The materials for the aligners were shaped into 3.5 mm diameter discs by punching both the original and thermoformed sheets. Following the International Organization for Standardization (ISO) standard 10993—12:2012, the ratio between the weight of the samples and the volume of the dilutions was 0.1 g/mL. Totally, calculate that each brand's original and thermoformed plastic sheets require 3.2 g for three different soaking days. The accomplished accurate weight for each brand's aligners were disinfected them by soaking in 75% alcohol. Following disinfection, immerse and rinse the discs twice in DMEM to eliminate any remaining disinfecting alcohol that could impact cell survival. Subsequently, use a 0.22 μm filter to purge any impurities from the DMEM. Continue to incubate the discs soaked in DMEM for seven and fourteen days in cell culture dishes. Assess the viability of the HPDL cells on days one, three, and five, while examining the cellular morphology under 100x, 200x, and 400x magnification using optical microscopes.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay

MTT assay (Sigma Aldrich, St. Louis, MO, USA) dye was used to evaluate cell viability. The 5×10^4 HPDL cells were planted into 24-well flat-bottomed, tissue culture plates. After 24 h of incubation, the culture medium was replaced with 200 lL/well of the extract. After additional 24 h, the medium was replaced with 100 lL/well of the MTT solution (1 mg/mL) in PBS, and the cells were incubated for an additional hour at 37 °C in a 5% CO_2 atmosphere. After the solution was removed, 100 lL/well of dimethyl sulfoxide was added and the plates were swirled gently for 10 min. The optical density of each well was immediately measured in a spectrophotometer (Sunrise, Team, Mannederf, Zurich, Switzerland) at 540 nm.

The following formula was used to calculate the cell viability: Cell viability (%) = (optical density of test group/optical density of cellular control group) x100.[6] The classification of cell viability was as follow: More than 90% cell viability showed no cytotoxicity, 60%—90% cell viability showed slight cytotoxicity, 30%—59% cell viability showed moderate cytotoxicity and less than 30% cell viability showed severe cytotoxicity.[7] To test cell viability, it is generally considered safe if the survival rate exceeds 70%.[8] Three independent experiments were performed in triplicate.After treatment with different materials, the morphology and number of HPDL cells were observed under a microscope at 100x, 200x, and 400x magnifications. The statistical analysis were performed using the statistical package for social sciences (SPSS 22.0, SPSS IBM, Armonk, NY, USA). Differences between mean values were

Table 1 Description of the ingredients and origins of the products used in this study.

Product brand	Composition	Company
DURAN®	Polyethylene terephthalateco-1, 4-cylclohexylenedimethylene terephthalate (PETG)	SCHEU-DENTAL *GmbH., Iserlohn*,Germany
ESSIX®	Polyethylene terephthalateco-1, 4-cylclohexylenedimethylene terephthalate (PETG)	Dentsply Sirona®, Fair Lawn, NJ, USA
Leone®	Poly ethylene terephthalateco-1, 4-cylclohexylenedimethylene terephthalate (PETG)	Leone S.p.a. Orthodontic and implantology, Firenze, Italy
Maxflex™	Thermoplastic polyurethane (TPU)	Cuumed Catheter Medical, Taichung, Taiwan
Zendura®	Thermoplastic polyurethane (TPU)	Bay Materials LLC, Fremont, CA, USA
Keystone®	Copolyester polyethylene terephthalate (PET)	Keystone® Industry, Gibbstown NJ, USA

determined by one-way of variance (ANOVA) with Bonferronni post-hoc test. The level of significance was set at $P < 0.05$.

Results

The viability of HPDL cells in PETG materials soaked for seven and fourteen days compared to a 70% performance benchmark is as follows:

The Duran experiment

Cell viability comparison in the fourteen-day immersed group: cell viability in the T-Duran group was lower than in the Duran group ($P < 0.05$). However, in the seven-day immersed group, cell viability in the Duran group was lower than in the T-Duran group ($P < 0.05$) (Fig. 1). Under the microscope, the HPDL cells were harvested on the first, third, and fifth days and examined at 100x, 200x, and 400x magnifications to assess cell morphology and count. The cells exhibited a spindle shape, and the number varied depending on the conditions (Fig. 2).

In Essix experiment

Cell viability comparison in the fourteen-day immersed group: cell viability in the T-Essix group was lower than in the Essex group ($P < 0.05$). However, in the seven-day immersed group, there was no difference in cell viability between the T-Essix and Essex groups ($P > 0.05$); both had survival rates above 70% (Fig. 3). The HPDL cells were harvested on the first, third, and fifth days, and examined under magnifications of 100x, 200x, and 400x microscope, the cells consistently exhibited a spindle shape, and the number varied under different conditions (Fig. 4).

In Leone experiment

Cell viability comparison in the fourteen-day immersed group: cell viability in the T-Leone group was lower than in the Leone group ($P < 0.05$). However, in the seven-day immersed group, there was a difference in cell viability between the T-Leone and Leone groups after five days of cell culture ($P < 0.05$), though both had survival rates above 70% (Fig. 5). The HPDL cells were harvested on the

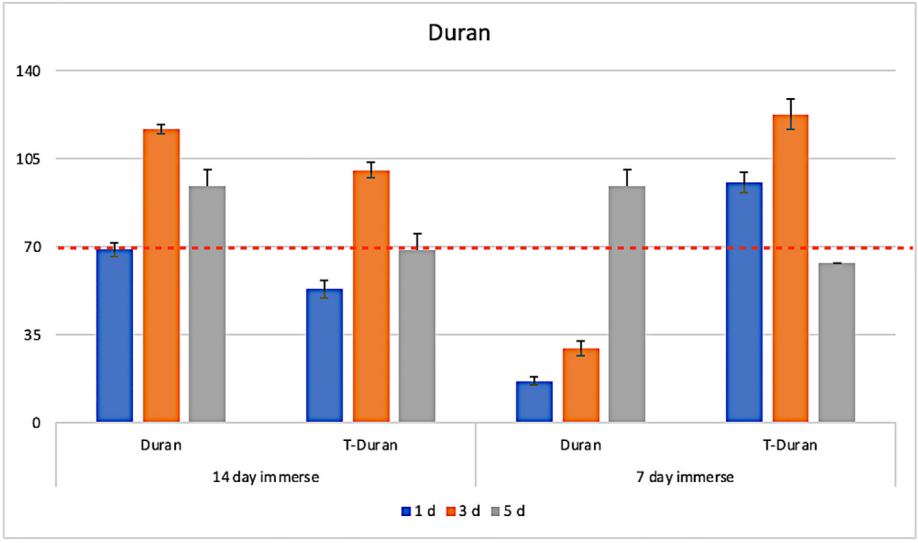

Figure 1 The viability of HPDL cells in Duran materials soaked for seven and fourteen days compared to a 70% performance benchmark. Duran: non-thermoformed. T-Duran: thermoformed. d: day. T: thermoformed.

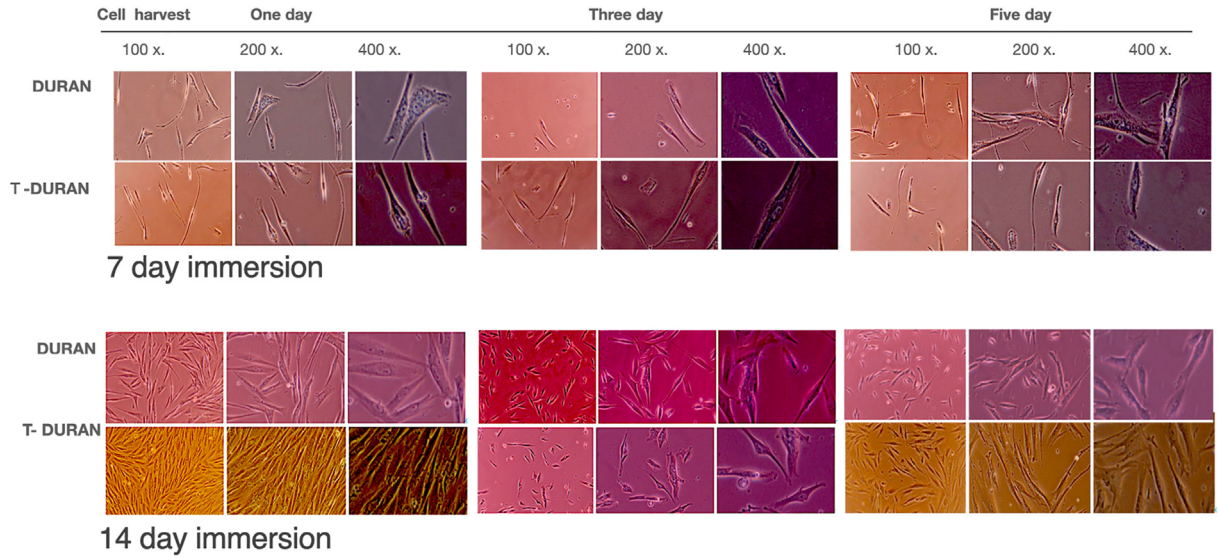

Figure 2 The interaction between HPDL cells and Duran and T-Duran membranes soaked for different durations. HPDL cells were collected on days one, three, and five, and examined at magnifications of 100x, 200x, and 400x to evaluate cell morphology and number. T: thermoformed.

first, third, and fifth days, and examined under magnifications of 100x, 200x, and 400x microscope, the cells consistently exhibited a spindle shape, and the number varied under different conditions (Fig. 6).

The performance of HPDL cell viability in TPU materials soaked for seven and fourteen days, compared to a 70% benchmark, is as follows:

In Zendura experiment

Cell viability comparison in the fourteen-day immersed group: on the first day of cell culture, cell viability in the Zendura group was lower than in the T-Zendura group ($P < 0.05$). In the seven-day immersed group, both the Zendura and T-Zendura groups had survival rates above 70%, with no differences between them ($P > 0.05$) (Fig. 7).

The HPDL cells were harvested on the first, third, and fifth days, and examined under magnifications of 100x, 200x, and 400x microscope, the cells consistently exhibited a spindle shape, and the number varied under different conditions (Fig. 8).

In Maxflex experiment

Cell viability comparison in the fourteen-day immersed group: on the first and fifth days of cell culture, cell viability in the Maxflex group was lower than in the T-Maxflex group ($P < 0.05$). In the seven-day immersed group, although there was a difference in cell viability between the Maxflex and T-Maxflex groups ($P < 0.05$), both had survival rates above 70% (Fig. 9). The HPDL cells were harvested on day 1, day 3, and day 5, and their morphology

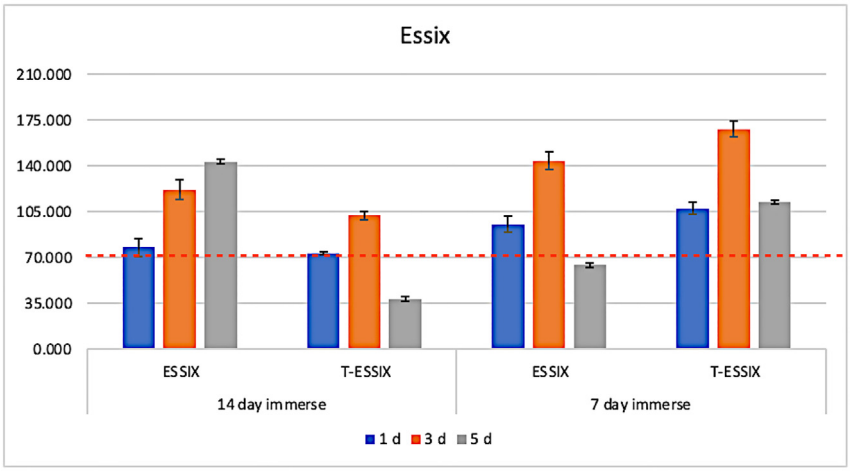

Figure 3 The viability of HPDL cells in Essix materials soaked for seven and fourteen days compared to a 70% performance benchmark. Essix: non-thermoformed. T-Essix: thermoformed. d: day. T: thermoformed.

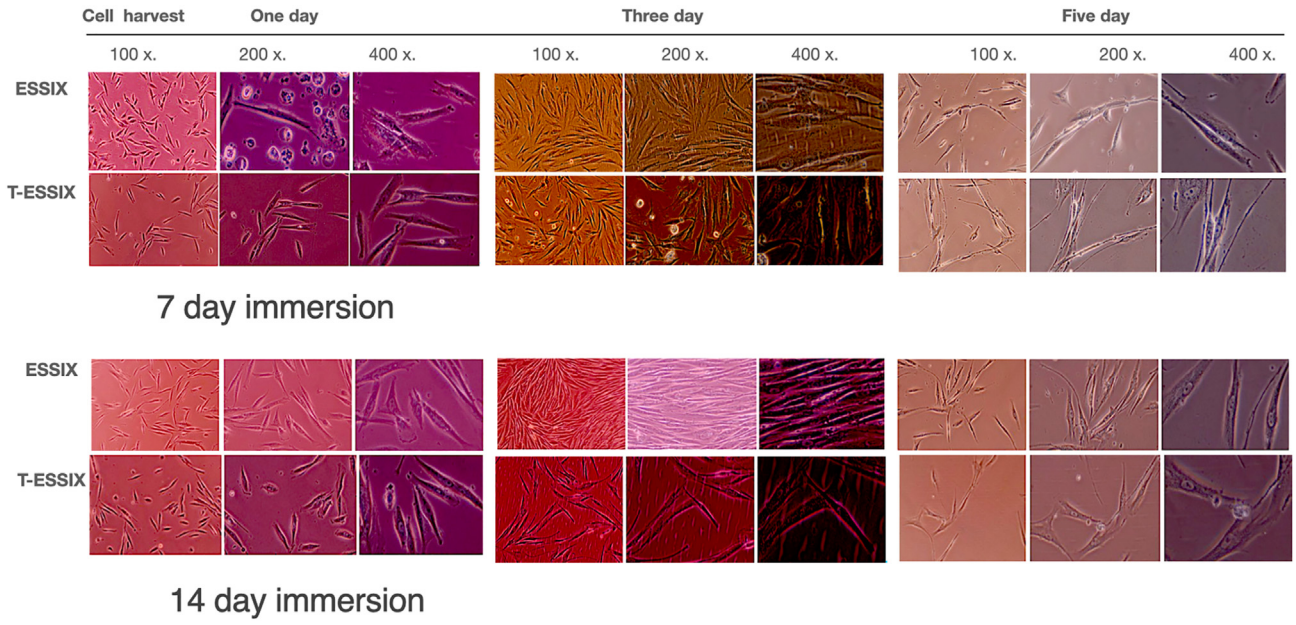

Figure 4 The interaction between HPDL cells and Essix and T-Essix membranes soaked for different durations. HPDL cells were collected on days one, three, and five, and examined at magnifications of 100x, 200x, and 400x to evaluate cell morphology and number. T: thermoformed.

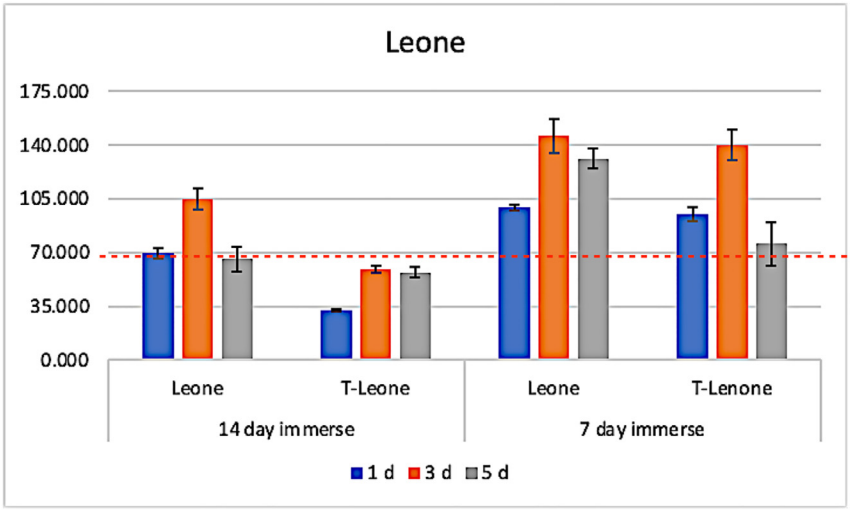

Figure 5 The viability of HPDL cells in Leone materials soaked for seven and fourteen days compared to a 70% performance benchmark. Leone: non-thermoformed. T-Leone: thermoformed. d: day. T: thermoformed.

and numbers were analyzed at magnifications of 100x, 200x, and 400x microscope, the cells were spindle-shaped, and the number varied under different conditions (Fig. 10).

The performance of HPDL cell viability in PET materials soaked for seven and fourteen days compared to a 70% benchmark is as follows:

In Keystone experiment

Cell viability comparison in the fourteen-day immersed group: on the first and fifth days of cell culture, cell viability in the Keystone group was lower than in the T-Keystone group ($P < 0.05$). In the seven-day immersed group, although there was a difference in cell viability between the Keystone and T-Keystone groups ($P < 0.05$), the survival rates still exceeded 70% (Fig. 11). The HPDL cells were harvested on day 1, day 3, and day 5, and their morphology and numbers were assessed at magnifications of 100x, 200x, and 400x microscopy, the cells consistently exhibited a spindle shape, and the cell count varied under different conditions (Fig. 12).

Discussion

In the current study, we evaluated the cytotoxic effects of both thermoformed and non-thermoformed aligner

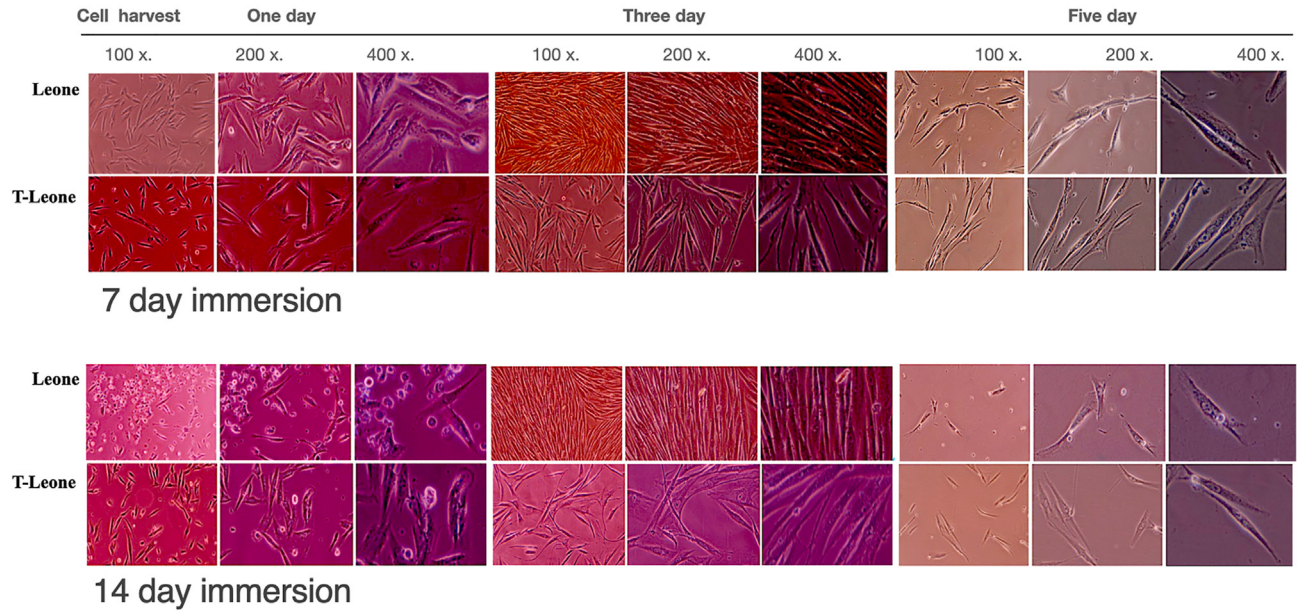

Figure 6 The interaction between HPDL cells and Leone and T-Leone membranes soaked for different durations. HPDL cells were collected on days one, three, and five, and examined at magnifications of 100x, 200x, and 400x to evaluate cell morphology and number. T: thermoformed.

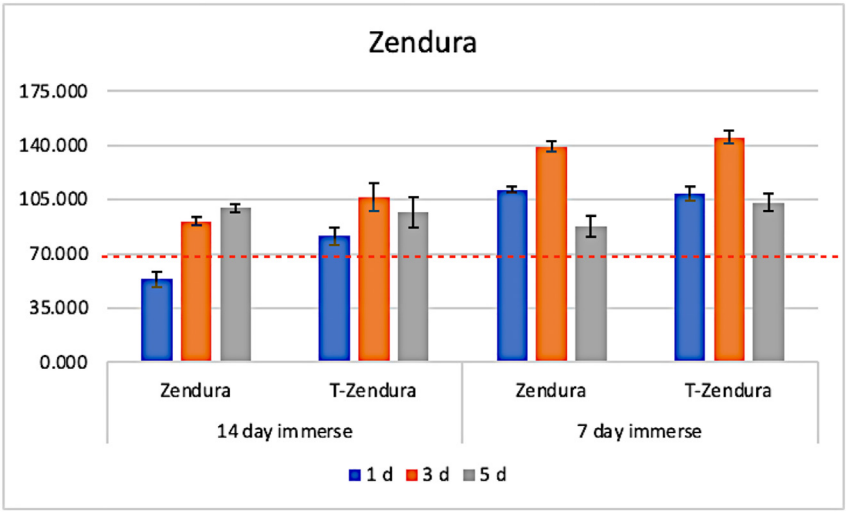

Figure 7 The viability of HPDL cells in Zendura materials soaked for seven and fourteen days compared to a 70% performance benchmark. Zendura: non-thermoformed. T-Zendura: thermoformed. d: day. T: thermoformed.

materials. Earlier research highlighted that certain materials did not retain their original properties in their non-heated, thermoformed state. For example, one study found that thermoformed materials were more cytotoxic. Among the materials tested, PETG (Biolon) showed the highest cytotoxicity to human primary gingival fibroblasts (HGFs) compared to TPU and PET. However, this study did not include the non-thermoformed multilayer aromatic thermoplastic polyurethane/copolyester (SmartTrack) material.[5] For present study, we utilized three primary types of materials: PETG, PET, and TPU, which are prevalent in the market and help to fill the research gaps identified in previous studies.

Contrary to previous findings, our study revealed that in the seven-day immersion test, thermoformed aligner materials maintained over 70% cell viability for PDL cells, indicating only slight cytotoxicity and performing better than non-thermoformed materials. In the fourteen-day test, except for the PETG (Duran, Essix, and Leone) group, which showed decreased survival rates in thermoformed materials compared to their non-thermoformed counterparts, thermoformed TPU and PET materials

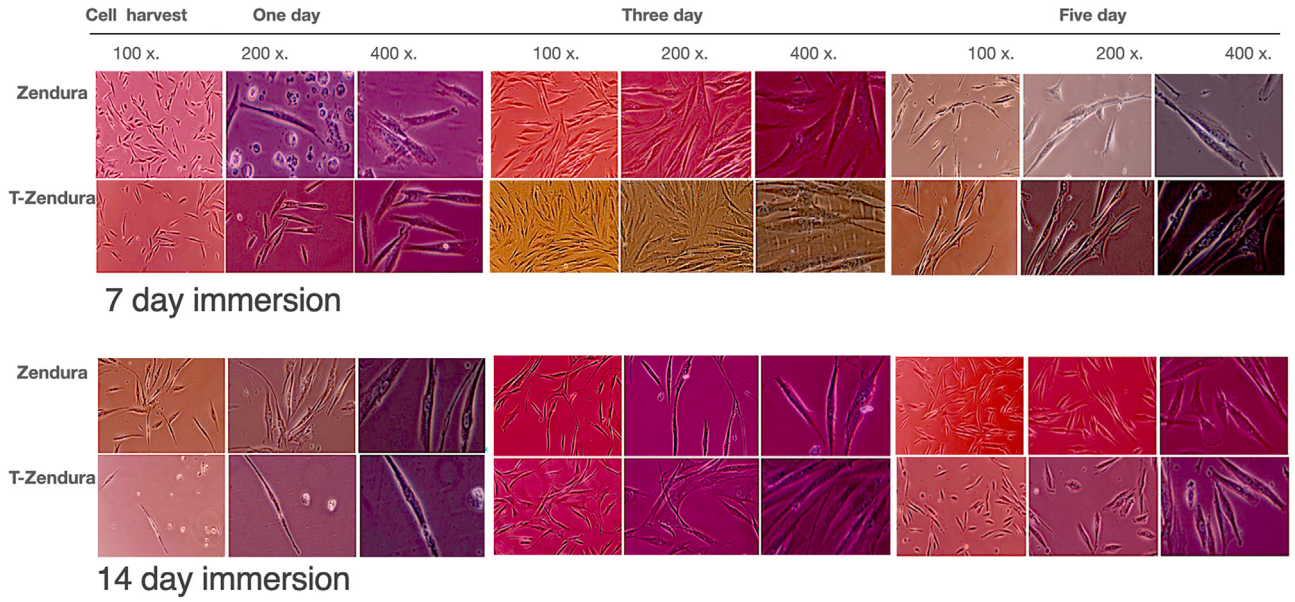

Figure 8 The interaction between HPDL cells and Zendura and T-Zendura membranes soaked for different durations. HPDL cells were collected on days one, three, and five, and examined at magnifications of 100x, 200x, and 400x to evaluate cell morphology and number. T: thermoformed.

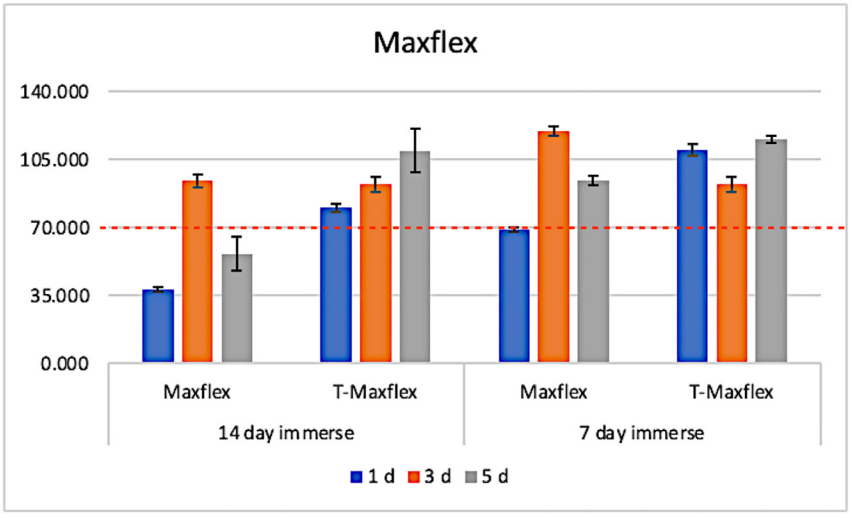

Figure 9 The viability of HPDL cells in Maxflex materials soaked for seven and fourteen days compared to a 70% performance benchmark. Maxflex: non-thermoformed. T-Maxflex thermoformed. d: day. T: thermoformed.

demonstrated higher viability rates than their non-thermoformed versions, maintaining over 70% viability and thus classified as slightly cytotoxic. Although previous research has proposed that thermoformed aligners might release monomers at elevated temperatures, contributing to cytotoxic effects, our study did not examine the release and concentration of these monomers, so we cannot confirm this hypothesis.

Additionally, the hygroscopic properties of PETG are suspected of facilitating ion release, potentially leading to cytotoxic effects.[9,10] PETG is noted for its superior abrasion resistance and ranks as one of the least hygroscopic commercially available materials. It is also frequently marketed as BPA-free. However, it is crucial to recognize that some PETG may incorporate BPA or similar compounds during its production, particularly when recycled PET is converted into PETG. This process could lead to the release of trace BPA amounts under specific conditions.

The hygroscopic nature of polymers could exacerbate cytotoxicity by causing ion release due to material degradation.[11] In our study, under the seven-day immersion conditions, both non-thermoformed and thermoformed materials showed only slight cytotoxicity with minor differences in viability for HPDL cells. Nevertheless, in the

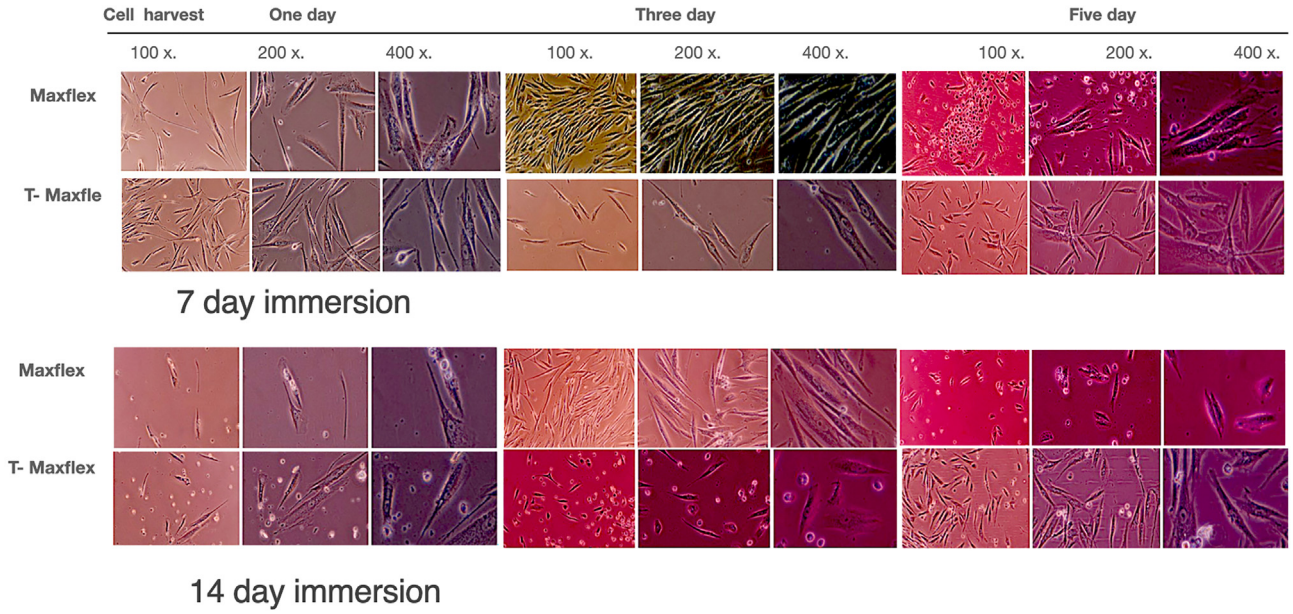

Figure 10 The interaction between HPDL cells and Maxflex and T-Maxflex membranes soaked for different durations. HPDL cells were collected on days one, three, and five, and examined at magnifications of 100x, 200x, and 400x to evaluate cell morphology and number. T: thermoformed.

fourteen-day immersion tests, non-thermoformed materials exhibited reduced viability for PDL cells in comparison to thermoformed materials. This decrease in cell viability could be attributed to the prolonged exposure leading to ion release from the hygroscopic characteristics of PETG.

Thermoplastic Polyurethane (TPU) is recognized for its flexibility, transparency, and oil resistance. It typically does not contain BPA, therefore not releasing BPA under standard conditions. TPU is mainly utilized in fields that require both flexibility and durability. In medical contexts, TPU has proven effective, extending beyond just aiding in bone and cartilage regeneration. Research involving a composite of TPU and polylactic acid (PLA) has shown that varying the TPU to PLA ratio significantly affects the proliferative and metabolic activities, cell adhesion, growth patterns, and cell—cell interactions of human adipose-derived mesenchymal stromal stem cells.[12] This confirms TPU's absence of BPA and its excellent biocompatibility. Accordingly, in our study, materials like Zendura and Maxflex that contain TPU consistently maintained cell viability above 70%.

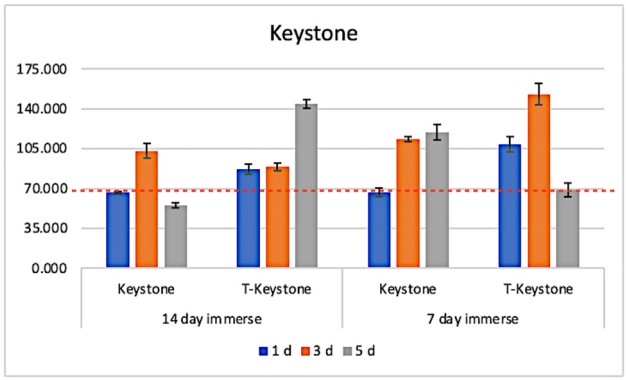

Figure 11 The viability of HPDL cells in Keystone materials soaked for seven and fourteen days compared to a 70% performance benchmark. Keystone: non-thermoformed. T-Keystone thermoformed. d: day. T: thermoformed.

PET (Polyethylene terephthalate) is the most commonly used polymer in plastic production, primarily for manufacturing beverage bottles and food containers. It is generally recognized as BPA-free and does not release BPA under normal usage conditions. However, PET can degrade when exposed to high temperatures or prolonged sunlight, potentially releasing small amounts of chemicals, including traces of BPA.[13] The viability of Human Embryonic Kidney-293 (HEK-293) cells and human immortalized keratinocytes (HaCaT) is influenced by exposure to PET microplastics, largely in a dose-dependent manner and varying by exposure time and cell type.[14] In contrast, an in vitro study examining the potential toxic effects of three nanoparticles—two generated by laser ablation (polycarbonate and one form of polyethylene terephthalate, PET1) and one (PET2) produced via nanoprecipitation—found no differentiated toxic effects in Caco-2 cells.[15] Further research indicates that nano-PET, at environmental concentrations, does not lead to significant toxicity endpoints in the respiratory system, as no evident cell apoptosis was observed.[16] In present study, the fourteen-day immersion group, the viability of thermoformed materials was only lower than that of the non-thermoformed materials after the first three days of culture. This finding aligns with previous research, suggesting that the heat treatment applied to PET does not adversely affect the viability of PDL cells. In conclusion, this study demonstrates that both thermoformed and non-thermoformed materials maintain high PDL cell viability rates, consistently above 70%, affirming their low toxicity under the conditions tested.

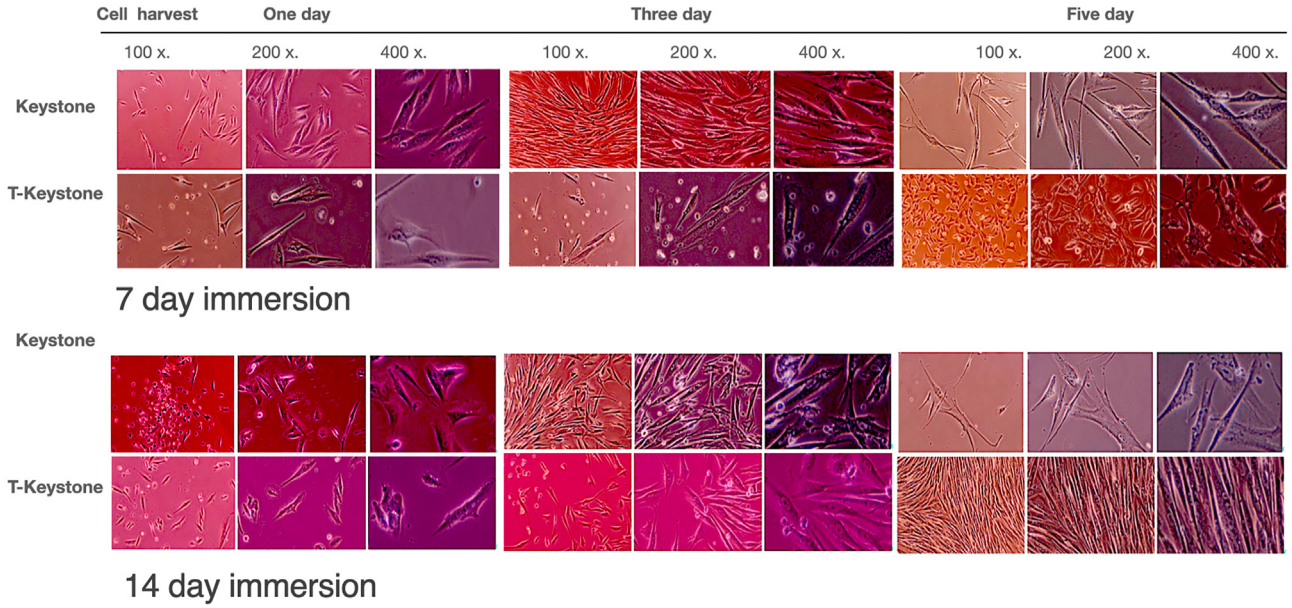

Figure 12 The interaction between HPDL cells and Keystone and T-Keystone membranes soaked for different durations. HPDL cells were collected on days one, three, and five, and examined at magnifications of 100x, 200x, and 400x to evaluate cell morphology and number. T: thermoformed.

Declaration of competing interest

All authors have no conflicts of interest relevant to this article.

Acknowledgments

This project was supported by grants from the Chung Shan Medical University Hospital (CSH-2024-C-058) Taichung Taiwan.

References

1. Bradley TG, Teske L, Eliades G, Zinelis S, Eliades T. Do the mechanical and chemical properties of Invisalign TM appliances change after use? A retrieval analysis. *Eur J Orthod* 2016;38:27—31.
2. Al Naqbi SR, Pratsinis H, Kletsas D, Eliades T, Athanasiou AE. In vitro assessment of cytotoxicity and estrogenicity of Viera retainers. *J Contemp Dent Pract* 2018;19:1163—8.
3. Nemec M, Bartholomaeus HM, Bertl HM, et al. Behaviour of human oral epithelial cells grown on Invisalign® SmartTrack® material. *Materials* 2020;13:5311—23.
4. Katras S, Ma D, Al Dayeh A, Tipton D. Bisphenol-A release from orthodontic clear aligners: an in vitro study. *Recent Prog Mater* 2021;3:1—11.
5. Martina S, Rongo R, Bucci R, Razionale AV, Valletta R, D'Ant 'o V. In vitro cytotoxicity of different thermoplastic materials for clear aligners. *Angle Orthod* 2019;89:942—5.
6. Vande Vannet B, Mohebbian N, Wehrbein H. Toxicity of used orthodontic archwires assessed by three-dimensional cell culture. *Eur J Orthod* 2006;28:426—32.
7. Ahrari F, Tavakkol Afshari J, Poosti M, Brook A. Cytotoxicity of orthodontic bonding adhesive resins on human oral fibroblasts. *Eur J Orthod* 2010;32:688—92.
8. Yashodhan M, Bichu YM, Alwafi A, et al. Advances in orthodontic clear aligner materials. *Bioact Mater* 2023;22:384—403.
9. Tamburrino F, D'Antò V, Bucci R, Alessandri-Bonetti G, Barone S, Razionale AV. Mechanical properties of thermoplastic polymers for aligner manufacturing: in vitro study. *Dent J* 2020;8:47—56.
10. Serra-Aguila A, Puigoriol-Forcada JM, Reyes G, Menacho J. Estimation of tensile modulus of a thermoplastic material from dynamic mechanical analysis: application to polyamide 66. *Polymers* 2022;14:1210—21.
11. Boubakri A, Elleuch K, Guermazi N, Ayedi HF. Investigations on hygrothermal aging of thermoplastic polyurethane material. *Mater Des* 2009;10:3958—65.
12. Lis-Bartos A, Smieszek A, Fra´nczyk K, Marycz K. Fabrication, characterization, and cytotoxicity of thermoplastic polyurethane/poly(lactic acid) material using human adipose derived mesenchymal stromal stem cells (hASCs). *Polymers* 2018;10:107—87.
13. Bittner GD, Denison MS, Yang CZ, Stoner MA, He G. Chemicals having estrogenic activity can be released from some bisphenol a-free, hard and clear thermoplastic resins. *Enviro Health* 2014;13:103—21.
14. Cui L, Digiacomo L, Xiao S, et al. Insights into the effect of polyethylene terephthalate (PET) microplastics on HER2 signaling pathways. *Toxicol Vitro* 2023;91:105632—7.
15. Tolardo V, Magrì D, Fumagalli F, et al. In vitro high-throughput toxicological assessment of nanoplastics. *Nanomaterials* 2022;12:1947—59.
16. Zhang H, Zhang S, Duan Z, Wang L. Pulmonary toxicology assessment of polyethylene terephthalate nanoplastic particles in vitro. *Environ Int* 2022;162:107177—92.