Original Article

In vitro cytotoxicity of different thermoplastic materials for clear aligners

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ABSTRACT

Objectives: To investigate the in vitro cytotoxicity of different thermoplastic materials for clear aligners on human primary gingival fibroblasts (HGFs).

Materials and Methods: Four materials for clear aligners were considered in this study: Duran (Scheu-Dental GmbH, Iserlohn, Germany), Biolon (Dreve Dentamid GmbH, Unna, Germany), Zendura (Bay Materials LLC, Fremont, CA, USA), and SmartTrack (Align Technology, San Jose, CA, USA). Three out of four materials (Duran, Biolon, Zendura) were assessed as thermoformed and nonthermoformed, whereas the SmartTrack was assessed only as thermoformed. The samples were placed at 37°C in airtight test tubes containing Dulbecco's Modified Eagle's Medium (DMEM; 0.1 mg/mL) for 14 days. The cell viability of HGFs cultured with this medium was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data were analyzed by means of one-way and two-way analysis of variance and post hoc tests ($\alpha = 0.05$).

Results: Each material exhibited a slight cytotoxic effect after 14 days. The highest cytotoxicity level on HGFs was achieved by Biolon (64.6% $\pm$ 3.3 of cell viability), followed by Zendura (74.4% $\pm$ 2.3 of cell viability), SmartTrack (78.8% $\pm$ 6.3 of cell viability), and finally Duran (84.6% $\pm$ 4 of cell viability), which was the least cytotoxic. In the comparison between nonthermoformed and thermoformed materials for Duran, Biolon, and Zendura, the thermoformed materials showed the highest level of cytotoxicity ($P < .001$).

Conclusions: Under the experimental conditions of this study, all the materials for clear aligners presented a slight cytotoxicity. Biolon was the most cytotoxic and the thermoforming process increased the cytotoxicity of the materials. (*Angle Orthod.* 0000;00:000–000.)

KEY WORDS: Clear aligners; Biocompatibility; Cytotoxicity; Orthodontics

INTRODUCTION

Increasing demands for esthetic orthodontic treatment, not only for adults[1] but also for adolescents and children,[2] have made clear aligner treatments more common. Clear aligners are removable trays made of plastic material that should be worn about 22 hours a day, for 10–14 days each, for the whole duration of therapy. Thus, they are in close contact with teeth, gingiva, and intraoral fluids for a long time. Clear aligners are used for increasingly complex and, consequently, longer treatments.[3,4] The plastic materials might be affected by changes due to the oral environment and then release molecules that could be dangerous for oral cells; for these reasons, the cytotoxicity of aligner materials should be investigated.[5]

The biocompatibility of orthodontic materials, such as composite, bonding materials, miniscrews, brackets, and archwires,[6–9] has been investigated in depth, but only two previous studies focused on the potential toxicity of the materials in clear aligners.[10,11] Hence, the

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cytotoxicity of clear aligners is still a topic of debate due to the lack of scientific literature and the conflicting results in the few available studies. Additionally, in the last decade, several new aligners appeared on the market and there is a need to assess the cytotoxicity of the different materials used by different brands.

Hence, the aim of this study was to determine the in vitro cytotoxicity of different thermoplastic materials for clear aligners on human primary gingival fibroblasts (HGFs). The null hypothesis was that there is no difference in the cytotoxicity induced by eluates from different materials.

MATERIALS AND METHODS

Materials, Chemicals, and Cells

Primary HGFs were obtained from oral surgical procedures in healthy 20- to 30-year-old patients with their informed consent under a protocol approved by the Ethical Committee of the University of Naples "Federico II" (N.226/14). Tissue fragments were washed twice in phosphate buffered saline (PBS, Carlo Erba Reagents, Milan, Italy) and transferred into tissue culture dishes, placed in a humidified 5% CO2 incubator, in Dulbecco's Modified Eagle Medium (DMEM, Carlo Erba Reagents), supplemented with 10% Fetal Bovine Serum (FBS, Carlo Erba Reagents), 2 mM glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C. After 10 days, fragments were removed, and released fibroblasts started proliferating. Once confluence was obtained, cells were washed with PBS and detached from the culture dishes using a brief treatment with trypsin/ethylenediaminetetraacetic acid for 5 minutes and then recultured until a confluent monolayer was obtained again.

Sample Preparation

Four materials for clear aligners, Duran (Scheu-Dental GmbH, Iserlohn, Germany), Biolon (Dreve Dentamid GmbH, Unna, Germany), Zendura (Bay Materials LLC, Fremont, CA, USA), and SmartTrack (Align Technology, San Jose, CA, USA), were considered in this study (Table 1). Three out of the four materials (Duran, Biolon, Zendura) were assessed as thermoformed and not thermoformed, while SmartTrack was tested only as thermoformed.

The samples were sterilized following the protocol defined by the International Standards Organization (ISO) 10993-5 norm (the method recommended by the manufacturer). The samples were immersed in DMEM for 14 days and stored under stationary conditions at 37°C in airtight test tubes. The ratio between the weight of the samples and the volume of the dilutions was 0.1 g/mL as recommended by the ISO parameters. For each clear aligner material, three samples were used and, after each release interval, the extracts were sterile-filtered to eliminate solid particles and then stored at -20°C until further use.

Table 1. Description of the Tested Materials

Brand	Material Composition	Supplier
Duran	Polyethylene terephthalate glycol (PETG)	Scheu-Dental GmbH
Biolon	Polyethylene terephthalate glycol (PETG)	Dreve Dentamid GmbH
Zendura	Polyurethane resin	Bay Materials LLC
SmartTrack	Multilayer aromatic thermoplastic polyurethane/ copolyester.	Align Technology

Cell Viability Assessment

MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma Chemical Co., Milan, Italy) was used to evaluate cell viability. HGFs were planted into 96-well flat-bottomed, tissue culture plates, with a density of 104 cells/well. After 24 hours of incubation, the culture medium was replaced with 200 μL/well of the extract. After additional 24 hours, the medium was replaced with 100 μL/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% CO2 atmosphere. After the solution was removed, 100 μL/well of dimethyl sulfoxide was added and the plates were swirled gently for 10 minutes. The optical density of each well was immediately measured in a spectrophotometer (Sunrise, Team, Mannederf, Zurich, Switzerland) at 590 nm. The optical density of the cells cultured in the DMEM medium without any clear aligner material sample extracts was used as a negative control for 100% cell viability and as a reference for the determination of the level of cytotoxicity in the assay; the Para rubber instead was used as a positive control.

According to Vande Vannet et al.,[12] the following formula was used to calculate the cell viability:

Cell viability (%) = (optical density of test group ÷ optical density of cellular control group) × 100.

Cell viability was then scored according to the classification of Ahrari et al.:[13]

- More than 90% cell viability: no cytotoxicity
- 60%–90% cell viability: slight cytotoxicity
- 30%–59% cell viability: moderate cytotoxicity
- Less than 30% cell viability: severe cytotoxicity

Three independent experiments were performed in triplicate.

Table 2. Descriptive and Inferential Statistics of Cell Viability

	Day 14	
Brand	Mean ± SD	Cytotoxicity
Duran	84.6 ± 4.02*** A[a]	Slight
Biolon	64.6 ± 3.31*** B	Slight
Zendura	74.4 ± 2.34*** C	Slight
SmartTrack	78.8 ± 6.35*** AC	Slight
P Value	<.001	

*** $P < .001$ indicates statistically significant differences compared with the negative control.

[a] Different letters in the subgroups indicate the statistically significant differences among materials.

Statistical Analysis

Descriptive statistics and statistical analysis were performed using the Statistical Package for Social Sciences (SPSS 22.0, SPSS IBM, Armonk, NY, USA). Normal distribution of the data was confirmed by the Shapiro-Wilk test. Differences between mean values were determined by one-way and two-way analysis of variance (ANOVA) with Bonferronni post-hoc test. The level of significance was set at $P < 0.05$.

RESULTS

The MTT results and the level of cytotoxicity at each time point for thermoformed and non-thermoformed clear aligner materials evaluated are shown in Tables 2 and 3. All the materials exhibited a slight cytotoxic effect after 14 days.

Among thermoformed materials, the highest cytotoxicity on HGFs was achieved by Biolon (64.6% ± 3.3 of cell viability) followed by Zendura (74.4% ± 2.3 of cell viability), SmartTrack (78.8% ± 6.3 of cell viability), and finally Duran (84.6% ± 4 of cell viability), which was the least cytotoxic. The positive control showed a cell viability of 12.5% ± 4.3. In the comparison between the nonthermoformed and the thermoformed materials for Duran, Biolon and Zendura, the highest cytotoxicity was present in the thermoformed group ($P < .001$).

DISCUSSION

The present study evaluated the in vitro cytotoxicity of thermoplastic materials used by different brands of clear aligners after 14 days. To assess the cytotoxicity on cell cultures of the media containing sample extractions, HGFs were selected because they are the principal cell line in the oral tissues, together with epithelial keratinocytes, and are clinically exposed to the potential toxic effects of thermoplastic materials because the aligners contact the gingiva when they are worn. HGFs are among the most used cells to assess biocompatibility of dental materials and they are recommended for in vitro evaluation of materials by the International Standards Organization (ISO).[6]

The exposure time interval was chosen because patients usually change aligners after 14 days. Under the experimental conditions, all the materials showed a slight cytotoxic effect on day 14, with comparable cell viability levels. These results were similar or lower than the cytotoxicity level achieved by many other dental materials such as metallic brackets and bands, miniscrews, or bonding materials.[6–8]

On the other hand, there were only two previous studies in the scientific literature about cytotoxicity of clear aligner materials. Eliades et al.[10] investigated the possible release of bisphenol-A (BPA) from Invisalign thermoplastic materials. It could produce estrogenic and cytotoxic effects, as demonstrated in other studies for adhesives, composite resins, and polycarbonate products.[14,15] The authors did not find estrogenic and cytotoxic effects on HGFs in contrast with the results of the current study in which slight cytotoxicity was observed. Nevertheless, it should be considered that the SmartTrack material[16] was tested, a thermoplastic polyurethane that, in 2012, replaced the previous material used in the article by Eliades et al.[10] The authors also reported that the chemical composition of Invisalign plastic did not have the necessary ingredients to release bisphenol-A.

Premaraj et al.[11] hypothesized that isocyanate, another component of Invisalign material, could affect oral health. Indeed, contact allergic reactions to isocyanate exposure have been reported.[17,18] This study tested cytotoxicity in vitro on epithelial keratinocytes. In agreement with the current study results, the authors found that exposure to Invisalign plastic caused changes in viability, membrane permeability, and adhesion of

Table 3. Descriptive and Inferential Statistics of Cell Viability

	Day 14				
	Thermoformed		Nonthermoformed		
Brand	Mean ± SD	Cytotoxicity	Mean ± SD	Cytotoxicity	*P* Value
Duran	83.7 ± 5.91*** A[a]	Slight	94.9 ± 5.59 A	Slight	.002
Biolon	67.0 ± 3.22*** B	Slight	89.1 ± 1.77** A	Slight	<.001
Zendura	73.7 ± 3.19*** C	Slight	66.9 ± 0.16*** C	Slight	.035

** $P < .01$, *** $P < .001$ indicate statistically significant differences compared with the negative control.

[a] Different letters in the subgroups indicate the statistically significant differences among materials. *P* value of the Analysis of Variance.

epithelial cells in a saline solution environment. They also demonstrated that saliva might offer protection.

No previous scientific research compared the cytotoxicity of different thermoplastic materials of clear aligners. In the current study, the thermoforming effect on the cytotoxicity of plastic materials for clear aligners was assessed. The thermoformed materials showed more cytotoxic behavior; however, only three materials were analyzed in a nonthermoformed state because it was not possible to obtain the nonthermoformed SmartTrack material. It was previously shown that the release of monomers by dental composite materials induced cytotoxicity,[19] so a similar mechanism of actions by the thermoplastic materials could be assumed. Considering that the polyethylene terephthalate released more added substances as the temperature increased,[20] it is possible that the thermoforming process could increase the release of monomers and consequently increase the cytotoxicity.

It certainly should be considered that the intraoral environment cannot be completely simulated with in vitro methodologies.[21] It must be emphasized that eluates were obtained under stationary conditions, while intraoral use of aligners requires that the appliances are washed every time they are removed from the mouth. Furthermore, intraoral aging affects the properties of thermoplastic materials of aligners,[22] which could also influence the biocompatibility. However, it is equally true that the results revealed a slight level of cytotoxicity that could be considered clinically irrelevant.

CONCLUSIONS

- In conclusion, under the experimental conditions, all materials for clear aligners showed slight cytotoxicity.
- The thermoforming process increased the cytotoxicity of Polyethylene terephthalate glycol materials.
- Since materials for clear aligners have only shown a slight level of cytotoxicity, their clinical use may be considered safe.

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