Original Article

Cytotoxicity assessment of different clear aligner systems: *An in vitro study*

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ABSTRACT

Objectives: To evaluate and compare the cytotoxicity of multiple clear aligner systems (Invisalign, Eon, SureSmile, and Clarity).

Materials and Methods: A cytotoxicity assessment was carried out by immersing three sets of aligners from the included four systems in normal saline for 1 month at 37°C. The solutions were then diluted to three different concentrations (5%, 10%, and 20% volume/volume). Gingival fibroblasts were exposed to the solution after being seeded to 96-well microplates for 48 hours, and the medium was substituted with an MTT solution (MTT: 3-[4,5-dime- thylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). Optical density was then measured to determine cell viability and evaluate cytotoxicity subsequently.

Results: Cytotoxicity comparison showed no statistically significant difference among the four included systems. However, when cell viability of each system was compared with the control, a significant difference was reported at the 10% and 20% solution concentrations. The Clarity system had the lowest toxicity across all solution concentrations.

Conclusions: The thermoplastic materials used by all tested systems (Invisalign, Eon, SureSmile, and Clarity) presented some degree of toxicity (slight to moderate), with statistically significant mean differences compared with the control. (*Angle Orthod.* 2022;92:655–660.)

KEY WORDS: Clear aligners; Thermoplastic; Cytotoxicity; Cell viability; Gingival fibroblast

INTRODUCTION

Orthodontic practice has undergone major technological advances and innovations.[1] One of these advances in the orthodontic armamentarium was the introduction of transparent plastic materials used in clear aligner therapy (CAT).[2] Thermoplastic clear overlay appliances have been used in the field of dentistry for decades in a variety of forms: retainers, night guards, temporomandibular joint disorder splints, and bleaching trays.[3] A clear aligner is a transparent (esthetically driven), removable tray that fits over all teeth in the dental arch, creating a three-dimensional force system. These devices aim to move the targeted tooth/teeth incrementally as the patient wears successive trays.[4] CAT has evolved because of the several advantages of these appliances compared with conventional approaches, including less clinical visits, less orthodontic emergencies, and improved esthetics in addition to better oral hygiene, comfort, and periodontal health.[5]

Transparent aligners are generally fabricated from high-grade thermoplastic polyurethane using the thermoforming process on conventional or digital models. However, the safety and biological nature of the polyurethane material has been an issue among dental professionals.[6] Studies reported changes in gingival cell viability, membrane permeability, and cell-to-cell adhesion, which led to reduced epithelial integrity and micro leakage attributed to continuous exposure to specific clear aligner materials for a considerable period of time.[7,8]

In 2014, Premaraj et al. concluded that the use of transparent aligners was associated with a wide variety of allergic reactions, ranging from a simple sore throat to full body rashes.[9] Despite the fact that most invisible aligners are similar polyurethane thermosetting polymeric products, some variations do exist among the

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aligner systems used by different companies. These differences are attributed to processing variations in manufacturing techniques that incorporate various additives and dimensional characteristics.[10]

The incomplete conversion of monomers into polymers results in residual monomers that may leach into oral cavity saliva and consequently cause adverse biological reactions to the tissues.[11] The harmful effects of the unpolymerized monomers in the polymeric biomaterials that are commonly used in the dental field are known to introduce toxic effects at both the tissue and cell levels, including gingival inflammation or irritation, immune reactions, apoptosis, or cell cycle disturbances. The concentration of unreacted monomers differs depending on the polymerization method. Polymerization method and powder–liquid ratio as well as storage time all play a role in the cytotoxicity of those materials.[12] Cytotoxicity is the process of determining the degree to which a specific agent causes destructive action to certain cells, that is, the likelihood of a substance to damage cells or cause apoptosis.[13]

Some dental materials and products are known to induce cytotoxic effects in oral tissues on contact.[14] Crucial to determining the biocompatibility of a product, cytotoxicity is tested in two ways: in vivo or in vitro. In vitro studies are more commonly used to study cytotoxicity; indeed, they are considered a screening approach for all new dental biomaterials intended for human use. The cytotoxicity of dental polymers specifically has been investigated over the years using gingival fibroblasts.[11,12]

Various types of clear aligner systems exist. Align Technology (San Jose, Calif) is considered the leading company in the clear aligner market, producing the world's most advanced clear aligner system (Invisalign) from SmartTrack material.[15] In addition, Eon Holdings (Amman, JO), established in 2011, claims their clear removable aligners were designed and produced from medical-grade polyurethane.[16] In 2018, 3M company (St Paul, MN) launched their own clear aligner system called Clarity, made from durable and virtually invisible material.[17] Lastly, SureSmile aligners were designed by Dentsply-Sirona (Charlotte, NC) in 2019 and are produced from Essix plastic, a thermoformed polyurethane material; the company claims that the material used is highly degradable.[18]

The dilemma facing CAT studies are their susceptibility to rapid technological changes regarding the materials and manufacturing techniques used. The results reported from studies therefore do not always coincide with the features of the aligners currently in use. This is attributed to the rapid improvement and evolution of different types of clear aligners and the time needed for collecting data, experimenting, analyzing, and publishing.[5] Only three published studies have investigated the cytotoxicity of clear aligners. Therefore, the aim of this study was to evaluate and compare the degree of cytotoxicity of the following four different clear aligner systems: Invisalign, Eon, Clarity, and SureSmile. The null hypothesis was that no difference in cytotoxicity would be detected among the four systems at the three solution concentration levels (5%, 10%, and 20% volume/volume).

MATERIALS AND METHODS

Three sets of aligners (maxillary and mandibular trays) were obtained from four manufacturers (Invisalign, Eon, Clarity, and SureSmile) for this experiment. The aligner manufacturing companies were blinded to rule out bias. Each set of appliances was placed in a glass container and immersed in a normal saline solution for 1 month at 37°C. Then, samples of the eluents were diluted to multiple volume percentages: 5%, 10%, and 20% volume/volume. To prevent culture medium dilution and, thus, adverse effects on cell physiology, the maximum immersion media concentration was 20% volume/volume. Afterward, gingival fibroblast strains were used to evaluate the cytotoxicity of the appliance materials. This study was approved by the Institutional Review Board, College of Medicine, King Saud University (Number E-20-4759 and College of Dentistry Research Center (CDRC) number PR0112).

Gingival Fibroblast Cultures

The gingival fibroblast (GF) cell lines received (ScienCell, Carlsbad, Calif) were placed in culture dishes containing Dulbecco's minimal essential medium (DMEM) Gibco-BRL (Paisley, UK) and supplemented with antibiotics and antimycotic (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone), in addition to non-essential amino acids and 15% fetal bovine serum (FBS) Gibco–Bethesda Research Labs (BRL; Paisley, UK). Incubation ran for 24 hours in an environment of 5% carbon dioxide and 85% humidity at 37°C to obtain monolayer cell growth. The fibroblasts were then subcultured using a trypsin-citrate solution (0.3%) at a 1:2 split ratio. Afterward, serial passaging was performed once a week using the same medium (DMEM) with 10% fetal bovine serum (FBS; Gibco-BRL). The cells were tested to ensure that they were mycoplasma free.

Cytotoxicity Assay

Cytotoxicity was estimated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, St Louis, Mo) according to the protocol followed by Eliades et al.[19] The GFs were seeded to

Table 1. Intraexaminer Reliability (Intraclass Correlation Coefficient)

	Intraclass Correlation	95% Confidence Interval		*F* Test With True Value 0			
		Lower Bound	Upper Bound	Value	*df*1[a]	*df*2[a]	*P* Value
Single measures	0.904	0.356	0.969	44.376	71	71	.00***
Average measures	0.95	0.525	0.984	44.376	71	71	.00***

[a] *df* indicates degree of freedom.
*** $P < .001$.

96-well flat-bottomed microplates at a density of approximately 50,000 cells/cm^2, added to a medium containing 10% FBS, and left for 18 hours to attach. After plating for 18 hours, the aligner serial immersion solutions (5%, 10%, and 20%) were introduced, and the incubation period ran for 48 hours based on the protocol. Afterward, the medium was substituted with a 50-μl MTT solution dissolved at a final concentration of 1 mg per mL in serum-free phenol red-free DMEM and placed on the shaking table at 150 rpm for 5 minutes to thoroughly mix the MTT. The plates were returned and incubated at 37°C for 3 hours to allow the MTT to metabolize. Then the MTT metabolic product (formazan) was suspended with 100% isopropanol and shaken for 5 minutes at 150 rpm to mix the formazan into the solvent. The reduced MTT was measured spectrophotometrically. Optical density (OD) was measured at a wavelength of 570 nm compared with a reference wavelength of 690 nm.[19]

Cell viability was calculated using the most common formula as follows:[20]

$$\text{Cell Viability } \% = \frac{OD_{570e}}{OD_{570b}} \times 100$$

OD_{570e} = mean value of the measured OD of the sample, and OD_{570b} = mean value of the measured OD of the control. The OD of the cells that were not exposed to an aligner extract and only cultured in DMEM media served as controls with a cell viability of 100%, a reference for cytotoxicity assessment. Cytotoxicity classification based on cell viability percentage occurred according to the following scoring technique previously introduced by Ahrari et al.:[21]

1. No cytotoxicity: cell viability more than 90%.
2. Slight cytotoxicity: cell viability 60%–90%.
3. Moderate cytotoxicity: cell viability 30%–59%
4. Severe cytotoxicity: cell viability less than 30%.

Statistical Analysis

The data analyses were carried out using Statistical Package for the Social Sciences software version 26.0 (IBM Inc., Armonk, N.Y.). The Shapiro-Wilk test was performed to determine the normal data distribution. Parametric testing was used because the data were normally distributed. Two-way analysis of variance (ANOVA) followed by one-way ANOVA and a Tukey post hoc test were performed to analyze the differences among the clear aligner systems (Invisalign, Eon, Clarity, and SureSmile) and solution concentrations (5%, 10%, and 20%). Student's *t*-test was used to compare the four included systems to the control. All assessments were carried out by one examiner and repeated twice (average values were used) to confirm reproducibility and reliability, which were calculated with the intraclass correlation coefficient. Results were considered statistically significant when $P \leq .05$.

RESULTS

The intraclass correlation coefficient between different time points (2 weeks apart) by the same investigator was high (0.90), indicating good intraexaminer reliability (Table 1). The two-way ANOVA demonstrated statistically significant differences among the different solution concentrations; however, the four systems included were not significantly different from each other (Tables 2 and 3). Figure 1 illustrates the pattern of cytotoxicity: when the solution concentration increased, cell viability decreased, and cytotoxicity increased with the exception of the Eon system. The Clarity system demonstrated a higher viability closer to the control at its three concentrations, whereas Eon aligners did not follow the same pattern as the other systems, with the 10% viability slightly less compared with the 20% viability (but not statistically different).

The Invisalign and SureSmile systems were significantly different between the 5% and 20% solution concentrations, whereas the Eon system was significantly different between the 5% and 10% solution concentrations (Table 3). When compared with the control, Invisalign cell viability showed a statistically significant difference at the three solution concentra-

Table 2. Differences Among Aligner Systems and Solution Concentrations

Source	Type III Sum of Squares	*df*	Mean Square	*F* Test	*P* Value
Systems	1672.000	3	557.333	1.970	.128
Concentration	8509.114	2	4254.557	15.041	.000***
System × Concentration	861.470	6	143.578	0.508	.800

*** $P \leq .001$.

Table 3. Comparison Among Different Solution Concentrations in Each System via One-Way ANOVA and Post Hoc Test[a]

System	Solution Concentration, %	N	Mean ± SD	*F* Test	*P* Value	95% Confidence Interval for Mean		Multiple Comparisons (Post Hoc Tukey HSD)		
						Lower Bound	Upper Bound	5%	10%	20%
Invisalign	5	6	82.6 ± 13.6	4.983	.022*	68.304	96.804	1	0.076	0.023*
	10	6	60.3 ± 8.8			51.103	69.494	0.076	1	0.811
	20	6	54.5 ± 23.1			30.240	78.682	0.023*	0.811	1
Eon	5	6	85.1 ± 18.3	4.485	.030*	65.901	104.381	1	0.033*	0.87
	10	6	54.8 ± 16.8			37.131	72.454	0.033*	1	0.865
	20	6	60.4 ± 20.7			38.649	82.081	0.87	0.0865	1
SureSmile	5	6	85.4 ± 16.4	5.704	.014*	68.186	102.626	1	0.226	0.011*
	10	6	70.7 ± 15.8			54.218	87.275	0.226	1	0.258
	20	6	56.8 ± 11.3			44.955	68.677	0.011*	0.258	1
Clarity	5	6	89.3 ± 15.0	1.898	.184	73.540	105.034	1	0.266	0.222
	10	6	72.9 ± 21.1			68.304	96.804	0.266	1	0.992
	20	6	71.8 ± 15.4			51.103	69.494	0.222	0.992	1

[a] SD indicates standard deviation; HSD, honestly significant difference.
* $P \leq .05$.

tions. The other three systems were only statistically significantly different at the 10% and 20% solution concentrations (Table 4). Invisalign and SureSmile demonstrated slight cytotoxicity, scoring at 5% and 10%, whereas they were moderate at 20% concentrations. On the other hand, Clarity showed slight cytotoxicity scoring in all solution concentrations. However, slight cytotoxicity scoring was recorded in Eon at 5% and 20%, whereas moderate scoring was detected at 10% as shown in Table 4.

DISCUSSION

The present study found that the thermoplastic materials currently used by the four clear aligner systems—Invisalign, Eon, Clarity, and SureSmile—displayed some degree of cytotoxicity, with no statistically significant difference observed among the four systems. On the other hand, within each system at the solution concentrations (5%, 10%, and 20% volume/volume), statistically significant differences were observed. A statistically significant difference was found in cell viability compared with the controls for all four systems. Clarity aligners showed the least toxicity among the different solution concentrations, with all concentrations exhibiting only slight toxicity (89.2%–71.7%). Invisalign and SureSmile showed slight toxicity for the 5% and 10% dilutions, whereas the higher concentration of 20% showed moderate toxicity. Interestingly, the Eon system showed moderate toxicity in the 10% concentration, but slight toxicity in the 5% and 20% concentrations.

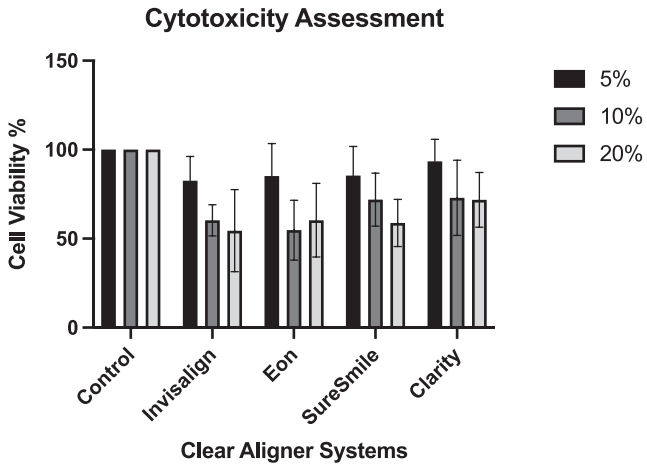

Figure 1. Cell viability assessment among different systems at various solution concentrations in comparison with the control.

The present findings were in agreement with a previous study conducted in 2019 that showed slight cytotoxicity in thermoplastic materials used by four different systems: Duran (Scheu-Dental GmbH, Iserlohn, Germany), Biolon (Dreve Dentamid GmbH, Unna, Germany), Zendura (Bay Materials LLC, Fremont, Calif), and SmartTrack (Align Technology). The authors suggested a possible correlation between the process of thermoforming and monomer release, which subsequently increased toxicity.[22] SmartTrack, used by the Invisalign system, is the only common material between the previous and present study. In addition, an older study reported unwanted cellular changes, including viability alterations (cytotoxicity), membrane permeability, and the adhesion of epithelial cells. It also suggested that saliva offered a protection mechanism against potential risks caused by thermoplastic materials.[9] However, other researchers reported contrasting results that did not reveal cytotoxic or estrogenic activity in Invisalign appliances based on laboratory assessments.[19] It should be emphasized that the original study was conducted in 2009 and, according to Align Technology, the currently used material replaced the previous material in 2012.[22]

GFs were selected for the current investigation because they are the principal cell line in oral tissues.

Table 4. Comparisons Between Different Clear Aligner System Cell Viabilities and the Control at the Three Solution Concentrations

		Test Value = 100							
System	Solution Concentration, %	N	Mean ± SD	*t*-Test	*P* Value	Mean Difference	95% Confidence Interval of the Difference		Cytotoxicity Score
							Lower	Upper	
Invisalign	5	6	82.6 ± 13.6	−3.147	.025*	−17.44610	−31.6957	−3.1965	Slight
	10	6	60.3 ± 8.8	−11.099	.001***	−39.70149	−48.8969	−30.5061	Slight
	20	6	54.5 ± 23.1	−4.833	.005**	−45.53897	−69.7599	−21.3180	Moderate
Eon	5	6	85.1 ± 18.3	−1.985	.104	−14.85904	−34.0988	4.3807	Slight
	10	6	54.8 ± 16.8	−6.58	.001***	−45.20730	−62.8685	−27.5461	Moderate
	20	6	60.4 ± 20.7	−4.692	.005**	−39.63516	−61.3512	−17.9191	Slight
SureSmile	5	6	85.4 ± 16.4	−2.179	.081	−14.59370	−31.8135	2.6261	Slight
	10	6	70.7 ± 15.8	−4.55	.006**	−29.25373	−45.7824	−12.7251	Slight
	20	6	56.8 ± 11.3	−9.359	.001***	−43.18408	−55.0448	−31.3234	Moderate
Clarity	5	6	89.3 ± 15.0	−1.749	.141	−10.71310	−26.4597	5.0335	Slight
	10	6	72.9 ± 21.1	−3.132	.026*	−27.03151	−49.2144	−4.8486	Slight
	20	6	71.8 ± 15.4	−4.501	.006**	−28.22554	−44.3461	−12.1050	Slight

* $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$.

GFs are exposed to potentially toxic effects from dental materials, especially aligners and retainers that come into direct contact with the gingival tissues. The International Standards Organization has recently recommended assessing dental materials through in vitro evaluation using the GF cell line.[23]

Further studies are needed under clinical conditions to evaluate the degree of toxicity intra-orally and to answer speculations about the protective mechanism of saliva, which might mitigate toxicity. Although the current study demonstrated some cytotoxic effects in the four clear aligner systems, their clinical usage should not be limited, as their toxicity was close to that previously reported in other orthodontic materials.[21,24,25] In addition, aligners are subjected to frequent changes, as the patient is required to use a new aligner every 7–10 days. Under the experimental conditions of the current study, all thermoplastic materials included from the four different clear aligner systems revealed some degree of cytotoxicity ranging from slight to moderate.

CONCLUSIONS

- The thermoplastic aligner materials from various systems (Invisalign, Eon, Clarity, and SureSmile) demonstrated slight to moderate cytotoxicity levels, with statistically significant differences compared with the control.
- No statistically significant differences were observed among the four systems.
- Lower solution concentrations had more cell viability and substantially less cytotoxicity.

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