Cytotoxicity and estrogenicity of a novel 3-dimensional printed orthodontic aligner

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Introduction: Orthodontic aligners printed with in-office 3-dimensional (3D) procedures have been described, but no data on their biocompatibility exist. This study investigates the cytotoxicity and estrogenicity of a 3D-printed orthodontic aligner by assessing its biological and behavioral effects. **Methods:** Ten sets of 1 type of aligner were immersed in sterile deionized water for 14 days, and the cytotoxicity and estrogenicity of released factors were assessed via MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays on human gingival fibroblasts and the estrogen-sensitive MCF-7 and the estrogen-insensitive MDA-MB-231 breast cancer cell lines. 17β-Estradiol and bisphenol-A were used as positive controls. The statistical analysis of data was performed with generalized linear models at a 0.05 level of significance. **Results:** No signs of cytotoxicity were seen for the aligner samples for concentrations (v/v) of 20% ($P = 0.32$), 10% ($P = 0.79$), or 5% ($P = 0.76$). The antioxidant activity expressed as the capacity to reduce intracellular levels of reactive oxygen species was not affected in the aligner samples ($P = 0.08$). No significant estrogenicity was induced by the aligner samples compared with eluents from the negative control for both MCF-7 ($P = 0.65$) and MDA-MB-231 ($P = 0.78$). As expected, 17β-Estradiol and bisphenol-A stimulated MCF-7 cell proliferation, whereas no effect was observed on MDA-MB-231 cells. **Conclusions:** In conclusion, if any factors were released during the 14-day aging of 3D-printed aligners in water, these were not found to be cytotoxic for human gingival fibroblasts and did not affect their intracellular reactive oxygen species levels. Moreover, no estrogenic effects of these putative eluates were observed based on an E-screen assay. (Am J Orthod Dentofacial Orthop 2022;162:e116-e122)

Orthodontic treatment of a large spectrum of malocclusions with aligners has become increasingly popular in recent years, partly because of the increased demand for treatment by adult patients and intense advertisement to patients. However, evidence about the objectively measured clinical performance of aligners compared with fixed appliances remains unclear.[1-4]

At the same time, orthodontic treatment with clear aligners involving the use of multiple, often bulky, composite resin attachments to enhance the aligners' clinical performance has introduced several issues[5] pertaining to alterations of the tooth structure or optical properties,[6-9] alterations of the aligners' material properties,[10-13] and alterations of the bonded resin attachments.[13,14] At the same time, intraoral aging of orthodontic materials affects their structural integrity and several material properties, like hydrolytic stability and plasticization,[4,5] which might result in component molecules being released intraorally, with bisphenol-A (BPA) being mostly discussed.

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The release of BPA from commercially available orthodontic aligners produced outside the office has not been definitively proven at the cell culture or analytical level. Previous laboratory studies of Invisalign aligners did not identify considerable cytotoxicity[15] or leaching of components.[16-18] In addition, no considerable alterations in the chemical composition of as-received and retrieved Invisalign aligners have been identified,[12] whereas the use of polypropylene and polyethylene vacuum-formed retainers (made in-laboratory or in-office) has been linked to increased salivary BPA levels.[18,19]

To avoid additional fees and delays to aligner delivery because of the involvement of third parties or companies, in-house 3-dimensional (3D) printing technology has been employed in the last years as a cost-effective do-it-yourself method to plan, produce, and deliver orthodontic aligners.[20-23] Although this technique looks promising, the current evidence base is limited. Currently, some first studies suggest that 3D-printed aligners have acceptable mechanical properties,[22] which might not be considerably altered by intraoral aging.[23] However, issues about their biocompatibility remain unanswered.

Therefore, the present in vitro study aimed to assess the cytotoxicity, antioxidative activity, and estrogenicity of contemporary 3D printed orthodontic aligners.

MATERIAL AND METHODS

The intraoral 3D scans of a patient were imported in Deltaface orthodontic computer-aided design software (Coruo, Limoges, France). The software performed a digital set-up, and the aligners were virtually designed accordingly. All the virtual aligners were imported into the Rayware software of the Sprintray Pro 55 3D printer (Sprintray, Los Angeles, Calif). Tera Harz TC85A aligner resin (Graphy, Seoul, South Korea) was used to print the aligners. After printing, the aligners were removed from the printer's platform and positioned in a centrifuge machine for 4 minutes. In the next step, the printing supports of the aligners were removed, and the aligners were positioned horizontally in the Cure M curing machine (Graphy, Seoul, South Korea) and cured for 24 minutes at the internal and external aligner sides. Ten sets of new aligners produced by 3D printing were received, corresponding to 10 patients (a total of 20 aligners; 1 maxillary and 1 mandibular arch per patient). Each aligner was cut into 3 pieces to minimize the liquid volume required for full immersion. The 6 pieces corresponding to each patient were pooled and immersed in a glass bottle containing 25 mL sterile deionized water, to be incubated for 14 days at 37°C. Three separate samples of water without an aligner were incubated under the same conditions in parallel to be used as negative controls. Occasional shaking (twice a day) was applied to all samples. At the end of the incubation, supernatants were collected, aliquoted (1 mL per aliquot), and kept at −80°C until further experimental use. Before any use in cell cultures, the osmolality of the samples was adjusted by adding NaCl to a final concentration (w/v) of 0.9%.

A human gingival fibroblast strain, previously developed from a healthy young donor, was used for assessing the possible cytotoxicity, as described previously.[15] Briefly, after overnight incubation of cells plated in 96-well, flat-bottomed microplates (25,000 cells/cm^2), the samples were added at a final concentration (v/v) of 20%, 10%, and 5%, and left for a 72-hour incubation. The final numbers of viable cells were estimated by a modification of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) method, as described.[15]

The intracellular reactive oxygen species (ROS) levels of human gingival fibroblasts with the highest noncytotoxic concentration of each sample were assessed on the basis of the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) method.[24,25] Briefly, cells were cultured until confluency in 96-well clear-bottomed black microplates and then were incubated overnight with samples diluted in serum-free medium at a final concentration (v/v) of 20%. DCFH-DA was added at a final concentration of 10 μM, and after 30 minutes, fluorescence emission was determined at 520 nm after excitation at 485 nm. Trolox (40 μM), a water-soluble vitamin E analog, was used as a control antioxidant.

The possible estrogenicity of the samples was assessed using the E-screen.[26] MCF-7 (estrogen-sensitive) and MDA-MB-231 (estrogen-insensitive) human breast adenocarcinoma cells were grown as previously described,[27] and subjected for 6 days to samples at a final concentration (v/v) of 20% in estrogen-depleted medium. The final numbers of viable cells were estimated by a modification of the MTT method, as described.[25,27] 17β-Estradiol (E2; 10-9 M) and BPA (10-8 M) were used as positive controls. All measurements were repeated 3 times for each sample to incorporate variability because of the method for all analyses.

Statistical analysis

Statistical analyses included initially descriptive statistics with mean and standard deviation after appropriate checks. Differences between the aligner samples and the negative controls (sterile deionized water) were checked with general linear models (with

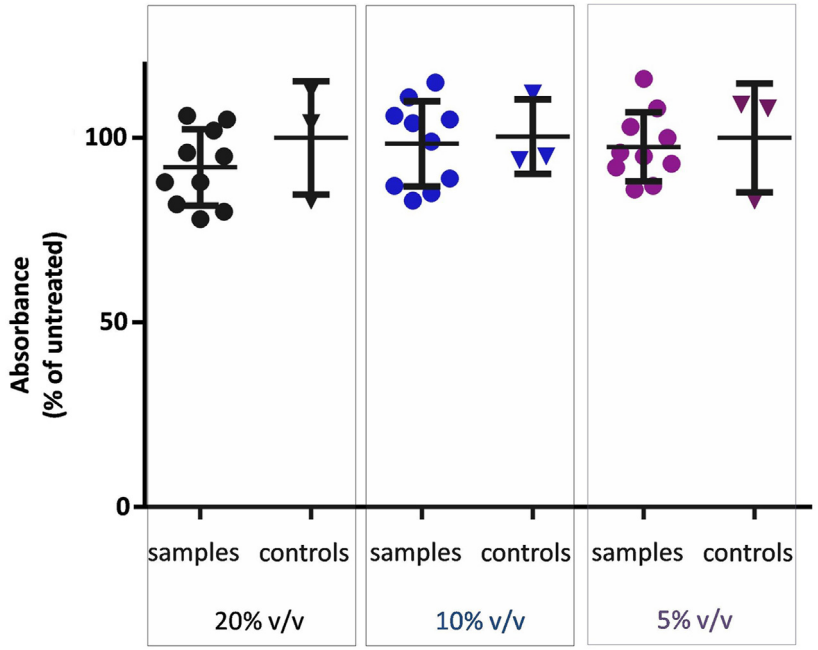

Fig 1. Viability of human gingival fibroblasts exposed to factors released during aging of 3D printed aligners.

adjusted errors for clustering within each sample) for cell viability (MTT method), antioxidant activity (ROS levels), and estrogenicity (E-screen assay) at an α of 5% in Stata (version 14.0; Stata Corp, College Station, Tex). Differences between the negative and positive controls (Trolox, E2, BPA) were checked to assess the responsiveness of the assays. The full dataset for all analyses was openly provided.[28]

RESULTS

As shown in Figure 1, the samples containing the 3D-printed-aligners' eluates were not cytotoxic for human gingival fibroblasts at all concentrations tested ($P = 0.32$ for 20% v/v; $P = 0.79$ for 10% v/v; $P = 0.76$ for 5% v/v).

The highest noncytotoxic concentration (ie, 20% v/v) was used to assess the samples' effects on the intracellular ROS levels of human gingival fibroblasts. As seen in Figure 2, all samples did not affect the ROS levels, whereas the well-known antioxidant Trolox suppressed ROS at 63.4% of the levels of the untreated cultures (Tables I and II; $P < 0.001$). Moreover, there was no statistical difference between the samples containing the 3D-printed-aligners' eluates and the negative controls ($P = 0.08$).

Finally, the 3D-printed-aligners' eluates at a final concentration of 20% v/v did not induce the proliferation of the estrogen-sensitive MCF-7 cells ($P = 0.65$), as depicted in Figure 3, whereas there was no difference between MCF-7 and MDA-MB-231 cell proliferation ($P = 0.78$). As expected, negative control samples also did not exhibit selective induction of MCF-7 proliferation. In contrast, in the positive controls, E2 and BPA induced MCF-7 proliferation at 165% and 140% of the control, respectively ($P < 0.001$ for both), without affecting MDA-MB-231 proliferation ($P = 0.07$ and $P = 0.66$, respectively), thus confirming the validity of this E-screen assay.

DISCUSSION

Cell culture systems of human origin are being extensively used for biocompatibility testing of materials used in dental practice because of their scientific relevance and advantages regarding ethical and financial issues.[29] Especially MTT analysis of cell viability has been used by us and others to assess the possible cytotoxic effects of substances released from dental materials and devices.[15,30,31] In this study, no difference was observed between the viability of human gingival fibroblasts exposed for 2 weeks to 3D-printed-aligners' eluates and those exposed to negative controls. Of course, this in vitro study focuses mainly on substances released by passive hydrolysis of the aligners and cannot take into account factors such as salivary enzymatic reactions,

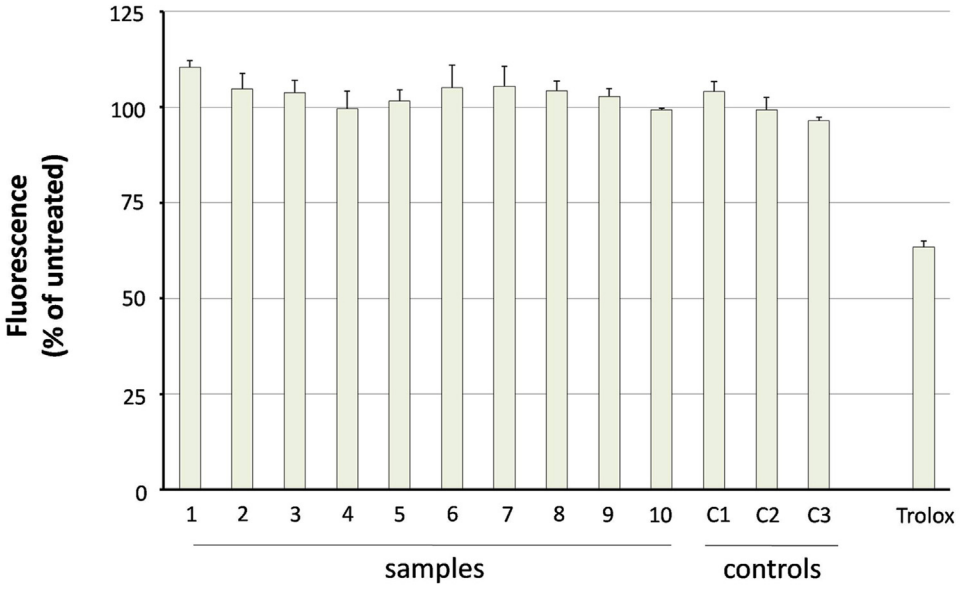

Fig 2. Intracellular ROS of human gingival fibroblasts after exposure to factors released during aging of 3D printed aligners.

Table I. Results of the cytotoxicity and estrogenicity assays, given as means ± standard deviation

Absorbance (% of untreated)	*Aligners*	*Negative control*	*NA*	*Trolox*	*E2*	*BPA*
Cell viability (MTT): 20% v/v	92.0 ± 13.0	100.0 ± 15.2	–	–	–	–
Cell viability (MTT): 10% v/v	98.3 ± 15.9	100.0 ± 11.0	–	–	–	–
Cell viability (MTT): 5% v/v	97.6 ± 14.6	100.0 ± 15.2	–	–	–	–
DCFH-DA assay	103.6 ± 4.4	99.9 ± 4.0	100.0 ± 4.2	63.4 ± 1.5	–	–
MCF-7 cells	70.6 ± 12.2	73.5 ± 26.1	100.0 ± 17.1	–	165.4 ± 24.1	140.0 ± 10.2
MDA-MB-231 cells	83.2 ± 9.8	85.4 ± 19.4	100.0 ± 7.9	–	100.6 ± 5.7	89.2 ± 6.4

NA, no addition.

chewing forces, dietary chemical effects, thermal changes, or effects of oral microbiota.[32] In contrast, this approach can compare these in-house produced 3D-printed aligners with other commercially available devices.[15,33]

Oxidative stress because of various materials used in conservative dentistry and orthodontics represents a major concern.[34] Although the 3D-printed-aligners' eluates were not cytotoxic for human gingival fibroblasts at all concentrations tested, we further tested the effects of the eluates on the oxidative status of these cells because we have previously observed the ability of a dental resin monomer to stimulate intracellular ROS at noncytotoxic concentrations in this cell type.[35] In this study, all 3D-printed-aligners' eluates and negative controls showed no stimulatory or inhibitory effects on intracellular ROS levels, as assessed by means of the cell-permeable fluorescent indicator DCFH-DA, whereas the well-known antioxidant Trolox, as expected, clearly suppressed ROS levels in human gingival fibroblasts.

Another aspect of biocompatibility tests regarding dental materials comprises their possible xenoestrogenicity.[26] By using an established E-screen assay that includes assessment of eluates on both estrogen-sensitive and estrogen-insensitive cells, we did not observe any xenoestrogenic activity eluted by 3D-printed aligners, in agreement with our previous observations from commercially available aligners.[15,33]

Hence, all assays in the current study support the idea that the specific resin used in this study for the 3D-printed aligner is biocompatible. A recent study assessing the cytotoxicity of 4 different contemporary thermoplastic materials for clear aligners on human gingival fibroblasts concluded that these materials might be slightly cytotoxic, especially after the thermoforming process; However, this toxicity could be considered

Table II. Statistical testing for the cytotoxicity and estrogenicity assays, given as *P* values from general linear models

	Aligner vs				*Negative control vs*		
Absorbance (% of untreated)	*Negative control*	*Trolox*	*β-estradiol*	*BPA*	*Trolox*	*β-estradiol*	*BPA*
Cell viability (MTT): 20% v/v	0.32	–	–	–	–	–	–
Cell viability (MTT): 10% v/v	0.79	–	–	–	–	–	–
Cell viability (MTT): 5% v/v	0.76	–	–	–	–	–	–
DCFH-DA assay	0.08	<0.001	–	–	<0.001	–	–
MCF-7 cells	0.65	–	<0.001	<0.001	–	<0.001	<0.001
MDA-MB-231 cells	0.78	–	<0.001	<0.001	–	0.07	0.66

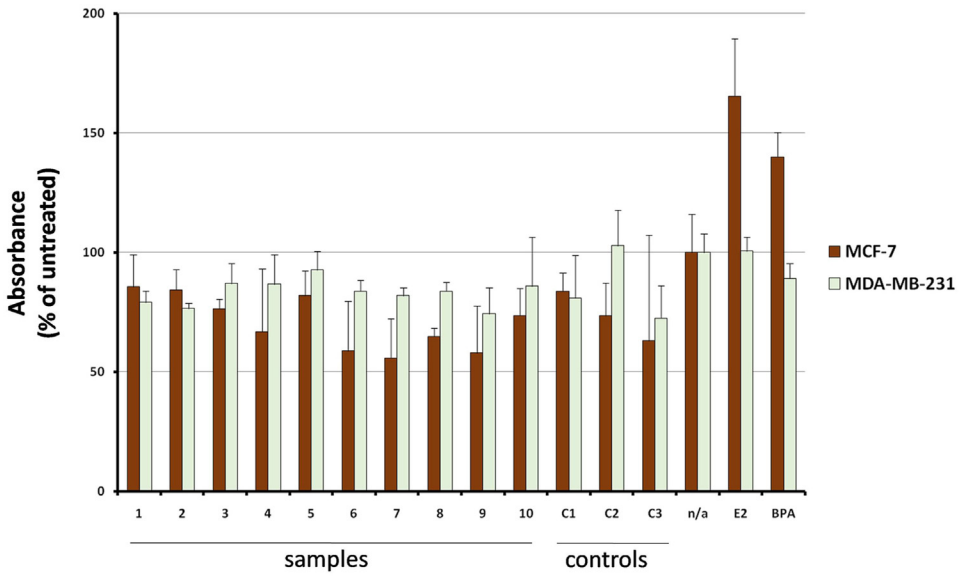

Fig 3. E-screen assay for detecting possible estrogenic factors released during aging of 3D printed aligners.

clinically irrelevant.[36] In contrast, 3D printing may be considered a more convenient alternative to the thermoforming process because it does not involve steps altering the material properties.[37]

A limitation of this study arises from the mode of application of aligners (conventional, thermoformed, and 3D-printed) in the clinical setting. The frequent (7-10 days) renewal of aligners introduces a variable that cannot be modeled in biocompatibility assays, as the continuous placement of new aligners in the oral cavity reinstitutes the source of elution in the oral cavity. Therefore, analysis of the release of constituents or by-products of aligners is necessary to establish the first pool of evidence regarding their reactivity with aqueous media to derive information about potential release patterns in the oral cavity.

The lack of information on the full synthesis of the aligner could have posed another obstacle in defining the justification of a potentially hazardous biological reactivity. Although there was no adverse effect identified in this investigation, it would be interesting to know that previous research has indicated that the resin used for the aligner tested is a vinyl-ester urethane-based resin.[23]

In addition, the oral environment constitutes a challenging environmental milieu for polymers owing to the presence of an aqueous environment with the concurrent presence of multiaxial loading during mastication; attrition and wear arising from the contact of the primarily softer aligner with the harder composite resin attachment, enzymatic activity including the effect of esterases on breaking down composite resins, microbial activity, pH, and temperature fluctuation. Therefore, in vitro assays, including aqueous immersion, underestimate the effect of environmental factors on the degradation potential of polymers. Thus, the results of studies involving materials used outside of their regular use fail to show the leaching status of compounds of potential biological reactivity.

CONCLUSIONS

If any factors were released during the aging of 3D printed aligners in water for 14 days, these were not found to be cytotoxic for human gingival fibroblasts and did not affect their intracellular ROS levels. Moreover, no estrogenic effects of these putative eluates were observed on the basis of an E-screen assay.

AUTHOR CREDIT STATEMENT

Harris Pratsinis contributed to methodology, investigation, validation, supervision, analysis, visualization, and manuscript review and editing; Spyridon N. Papageorgiou contributed to methodology, analysis, original draft preparation, and visualization; Nearchos Panayi contributed to conceptualization, methodology, investigation, funding acquisition, project administration, resources, original draft preparation, and manuscript review and editing; Anna Iliadi contributed to conceptualization, methodology, investigation, validation, and manuscript review and editing; Theodore Eliades contributed to conceptualization, methodology, investigation, supervision, funding acquisition, project administration, resources, original draft preparation, and manuscript review and editing; Dimitris Kletsas contributed to methodology, investigation, validation, supervision, analysis, visualization, and manuscript review and editing.

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