

Comparison of the cytotoxicity of 3D-printed aligners using different post-curing procedures: an in vitro study

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Objective: Three-dimensional (3D) printing technology represents a novel method for manufacturing aligners. The aim of the present study was to assess the in-vitro cytotoxicity of 3D-printed aligners using different post-polymerisation conditions.

Materials: Aligners were printed using the same 3D-print resin (TC-85DAC, Graphy, Seoul, Korea) and printer (AccuFab-L4D, Shining 3D Tech. Co., Hangzhou, China), followed by different post-curing procedures. Six aligners were post-polymerised for 14 min using the Tera Harz Cure and a nitrogen generator curing machine (THC2, Graphy, Seoul, Korea) (P1). A further six aligners were post-cured for 30 min on each side using the Form Cure machine (FormLabs Inc, Somerville, USA) (P2). The aligners were cut into smaller specimens (2 mm×2 mm) and sterilised at 121°C. The specimens were placed in 96-well plates containing Dulbecco's Modified Eagle's Medium (DMEM) at 37° for 7 or 14 days. The viability of MC3T3E-1 pre-osteoblasts cultured with DMEM was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The optical density of each cell culture was measured to assess cell viability, following which the data were statistically analysed using two-way and one-way ANOVA ($\alpha = 0.05$).

Results: The comparison of cytotoxicity revealed statistically significant differences between post-curing procedures and MTT timings ($P < 0.001$). After 7 and 14 days, the cell viability of P2 was significantly reduced compared to P1 and the control groups ($P < 0.001$), while P1 showed no significant differences compared to the controls. Overall, P2 post-curing exhibited moderate cytotoxicity, while P1 post-polymerisation was highly biocompatible.

Conclusions: Different post-curing procedures may affect the in-vitro cytotoxicity of 3D-printed aligners. Clinicians should adhere to the manufacturer's recommendations when using 3D-print resin.

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Introduction

The rapid technological advancements in recent years have strongly influenced orthodontic practices, particularly regarding the materials and manufacturing techniques employed by clinicians.¹ A significant innovation has been the utilisation of

thermoplastic materials for Clear Aligner Treatment (CAT). Although the clinical efficacy of CAT in achieving all types of tooth movements is still under debate,²⁻⁴ CAT has become a common treatment option for a wide range of orthodontic malocclusions, especially in mild-to-moderate cases.⁵

Despite the numerous advantages over traditional fixed appliance treatments, the growing success of CAT can be largely attributed to the aesthetic satisfaction of patients and the extensive promotional policies by companies, manufacturers, and stakeholders.⁶

Clear aligners are transparent removable trays which fit over dental structures, creating a three-dimensional (3D) force system to gradually move the target teeth to their planned positions.¹ Consequently, the biocompatibility of the materials used for aligners is an essential requirement, due to their continuous contact with the oral environment.⁷

Standard materials for the manufacture of aligners are polyurethane polymers and polyethylene glycol terephthalate (PETG) materials,⁸ which undergo a thermoforming process on physical models.⁷

For conventionally manufactured products, two previous studies which analysed polymer surface composition, bacterial adhesion, and biologic reactions in-vitro and in-vivo assessed the cytotoxicity, as well as the release of molecules from the aligners due to intraoral aging.^{8,9}

Despite the use of similar materials, some variations exist between aligners commercialised by different companies.¹ Martina et al.⁷ and Alhendi et al.¹ reported slight-to-moderate toxicity when testing multiple clear aligner systems, while Eliades et al.¹⁰ found no evidence of cytotoxicity of human gingival fibroblasts following Invisalign use. By evaluating different brands, Martina et al.⁷ also demonstrated that the thermoforming procedure may increase the in-vitro cytotoxic effects of various thermoplastic materials on human primary gingival fibroblasts.

Recently, alongside conventional fabrication using thermosetting polymeric products, an alternative method of aligner manufacture has emerged based on 3D-printing technology. Of the various available 3D-printing technologies in orthodontics, a commonly used process is stereolithography (STL).^{11,12} In this process, the photo-polymerisation of a photo-sensitive liquid resin enables the production of a solid object which, after 3D-printing, is exposed to an additional ultraviolet (UV) light source to complete the polymerisation reaction (post-curing process).¹²

As reported in the literature, the direct 3D-printing of aligners may reduce the negative effects of the thermoforming process,¹² which limits the geometric and dimensional distortions of the thermoplastic materials,¹³ while increasing the accuracy and efficacy

of the aligners.¹⁴ Moreover, the higher precision and customisable intra-aligner thickness improve the effectiveness of aligner production, by reducing the negative effects on the environment due to plastic disposal and carbon emissions.¹⁵ In addition, this innovative approach also allows for the planning, fabrication, and direct delivery of 3D-printed aligners directly in-office, therefore avoiding the involvement of third parties and resulting in cost and time-effectiveness.¹⁶

Although the mechanical characteristics of 3D-printed aligners have been previously evaluated,^{17–19} limited data are currently available regarding the biocompatibility of 3D-printed aligners.¹⁶ Pratsinis et al.¹⁶ assessed the biocompatibility and the absence of oestrogenic effects of the 3D-print polymer and showed that the elements released from 3D-printed aligners were cyto-compatible for human gingival fibroblasts. However, a lack of knowledge persists regarding the potential toxicity of the printable resin when using different post-polymerisation procedures. Post-polymerisation is the process by which the deposited material is cured following 3D-printing.¹⁵ This is a mandatory step for improving aligner mechanical properties¹⁷ and enhancing the biocompatibility of 3D-print resins.²⁰ Furthermore, different post-curing procedures may influence the levels of cytotoxicity because the level of conversion of 3D-photopolymer material might affect the biocompatibility of the resin.²⁰

Therefore, since the properties of 3D-printed aligners depend on post-curing parameters,⁸ investigating the selection of the most appropriate conditions for post-polymerisation is necessary for their appropriate application in orthodontics.

Therefore, the aim of the present study was to assess the cytotoxicity of 3D-printed material and determine if different post-curing processes influence in-vitro cytotoxicity. The null hypothesis was that different post-polymerisation conditions of the same 3D-print material would not exhibit any differences in in-vitro cytotoxic effects.

Materials and methods

Material preparation

An a priori power analysis was conducted using G*Power (Heinrich Heine Universität, Düsseldorf, Germany) version 3.1.9.6 to determine the minimum sample size required to test the study hypothesis.²¹ The results indicated that the required sample size to

achieve 95% power for detecting a medium effect, at a significance criterion of $\alpha = 0.05$, was $N = 12$.

Intra-oral scans were acquired using an intra-oral scanner (Trios3; 3Shape, Copenhagen, Denmark). The digital model's data were imported into orthodontic imaging software (OnyxCeph; Image Instruments, Chemnitz, Germany) (Figure 1). A digital set-up was performed, and the aligners were designed at a thickness of 0.5 mm (Figure 2). The final .STL file format compatible for 3D-printing was generated and imported into slicing software (AccuWare, Shining 3D Tech. Co., Ltd., Hangzhou, China) to manually insert the printing supports (Figure 3).

The aligners were printed by the same 3D-printer (AccuFab-L4D, Shining 3D Tech. Co., Ltd., Hangzhou, China) using Tera Harz TC-85 DAC resin (Graphy, Seoul, Korea), which is the polymer specifically marketed for the 3D-printing of orthodontic aligners. Twelve aligners were printed during three different 3D-printing cycles (Figure 4) and was performed at a printing orientation of 45° to the building platform, using a layer thickness of 100 μm .

The aligners were detached from the printer's platform and placed in a centrifuge machine for 6 min. Subsequently, the aligners were dried using compressed air to remove any excess resin accumulated during the post-curing process.

The printing supports were removed from the aligners, and the samples were post-cured under different conditions. Six aligners were placed horizontally in a Tera Harz Cure with a nitrogen generator (THC2) curing machine (Graphy, Seoul, Korea) and post-polymerised for 14 min (Postcuring

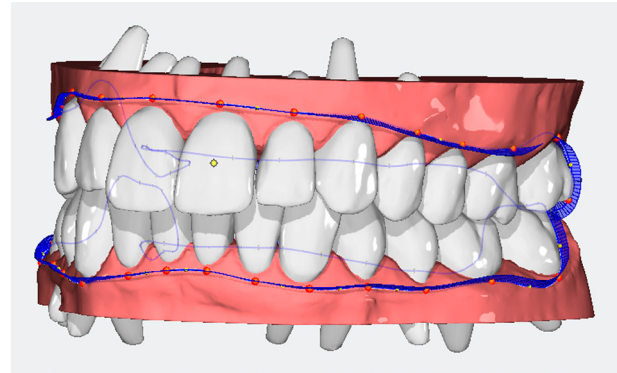


Figure 2. Digital design of the aligner.

1, P1). A second set of six aligners was post-cured for 30 min on each side of the aligner (totaling 60 min), using the *Form Cure* (FormLabs Inc, Somerville, USA) (Postcuring 2, P2).

The technical properties of the curing machines are summarised as follows:

- THC2: Light Source = UV LED; LED Wavelength = 405 nm; LED power Output = 200 W; LED Operation Temperature = 5 to 35°C.
- FormCure: Light Source = 13 multi-directional LEDs; LED Wavelength = 405 nm; LED power Output = 39 W; LED Operation Temperature = suggested 18° to 28°C (maximum 80°).

The aligners were washed in an ultrasonic cleaning machine at 80° for 2 min and then in boiling water for 1 min. After the washing procedures, the aligners were dried for 5 min using a drying machine. The final 3D-printed aligner is shown in Figure 5.

All of the aligners were cut into smaller specimens (2 mm×2 mm) and sterilised at 121°C.

Cytotoxicity testing

Pre-osteoblast mouse calvaria MC3T3E-1 cells (#CRL-2593, American type culture 257 collection, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (4.5 g/L D-Glucose, L-Glutamine, Pyruvate) supplemented with 10% fetal bovine serum and 1 X Gibco® Antibiotic-Antimycotic (Thermo Fisher Scientific, Waltham, MA, USA) in 96-well plates (9×10³ cells/well) at 37 °C in a humidified environment of 5% CO₂ and 95% air.

As reported by Park et al.,²² the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

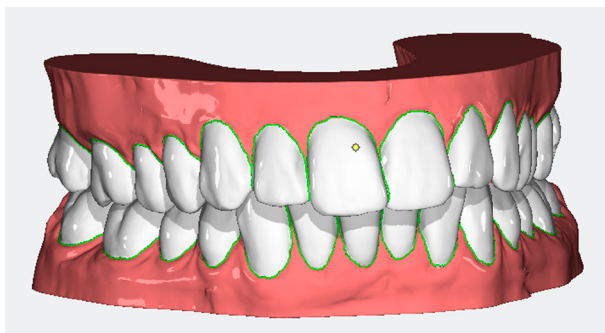


Figure 1. Digital models imported into the software.

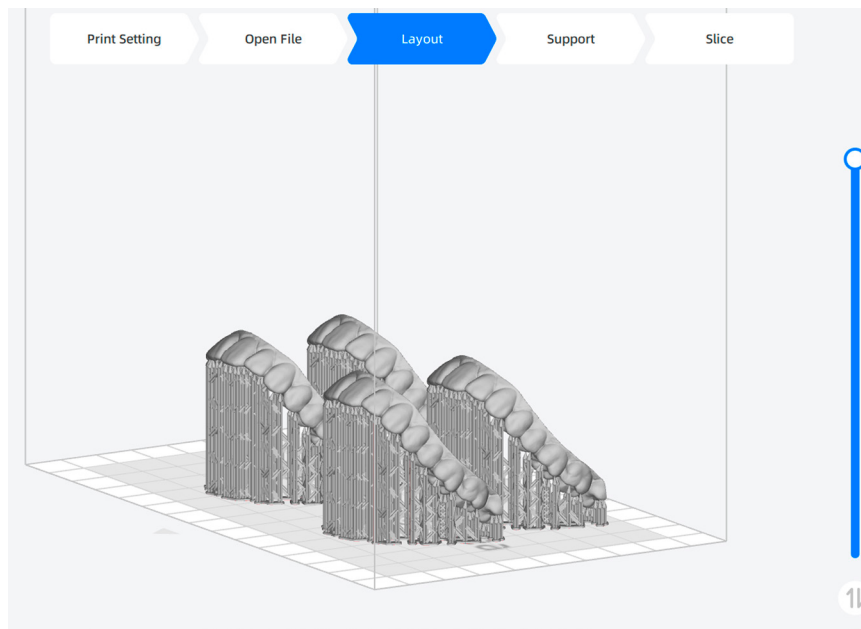


Figure 3. Printing supports.

was used to assess cell viability. Briefly, the cultures were washed with PBS-EDTA, the cells treated with an MTT solution (diluted 1:10 in DMEM) and incubated for 4 hrs, following which the formazan was solubilised using 100% DMSO.

The optical density (OD) of each well was measured using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 595 nm. The measurements were taken at 15 and 30 min after each test.

Two independent experiments were performed after 7 and 14 days, and both were performed in four triplicates:

- P1 = resin post-cured under P1 condition.
- P2 = resin post-cured under P2 condition.
- Positive controls (C+) = the DMEM medium with cultured cells, without any aligner specimens.
- Negative controls (C-) = the DMEM medium without cultured cells and aligners specimens.

To eliminate optical interference from background components, the values obtained for the DMEM (C- group) were subtracted by the means of the other groups (P1, P2, C+).

The OD of the C+ cells served as a reference for 100% cell viability and was used to assess the level of cytotoxicity in the assay.

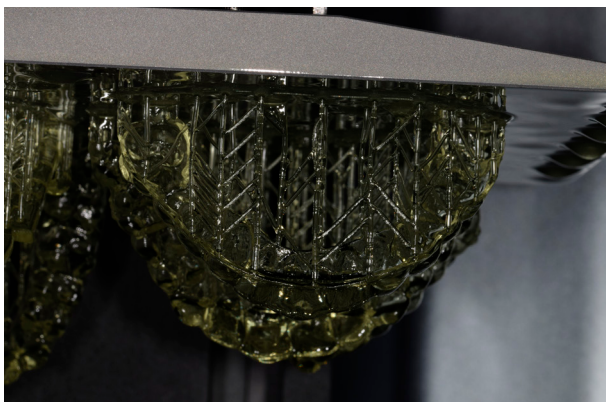


Figure 4. Printing process.



Figure 5. Final 3D-printed aligner.

Table I. Differences among groups (P1, C+, P2) and MTT timings (7 and 14 d).

Source	Type III sum of squares	df	Mean square	F test	P value
Group	7.374	2	3.687	166.341	<0.001 ***
MTT	4.978	1	4.978	224.604	<0.001 ***
Group*MTT	1.444	2	0.722	32.567	<0.001 ***

*** $P \leq 0.001$.

As previously reported,⁷ cell viability was calculated according to the following formula:

Cell viability (%) = (mean value of the OD of the examined groups/mean value of the OD of the control group) $\times$ 100.

According to Ahrari et al.,²³ the cytotoxicity was calculated as follows:

- No cytotoxicity = cell survival more than 90%.
- Slight cytotoxicity = cell survival between 60% to 90%.
- Moderate cytotoxicity = cell survival between 30% to 59%.
- Severe cytotoxicity = cell survival less than 30%.

Statistical Analysis

Descriptive statistics and a statistical analysis were performed using the Statistical Package for Social Sciences (SPSS 22.0, SPSS IBM, Armonk, NY, USA). The Shapiro–Wilk test was performed to assess the normal distribution of the data. Due to a normal distribution, parametric tests were applied. Two-way analysis of variance (ANOVA) and one-way ANOVA with Tukey's post hoc test were performed to analyse the differences between the groups (P1, C+, P2) and MTT timing (after 7 and 14 d). The level of significance for all analyses was set at $P \leq 0.05$.

Results

The results of the cytotoxicity assay and the level of cell viability for the P1, C+, and P2 groups after 7 and 14 days are shown in Tables I and II.

Statistically significant differences between the groups and the MTT timings were demonstrated using the two-way ANOVA (Table I). At 7 and 14 days, the P2 group exhibited significantly lower values compared to the P1 and the C+ groups ($P < 0.001$). At each time point, the cell's survival was significantly reduced in the P2 samples compared to the P1 samples ($P < 0.001$). A slight increase in cell viability was observed for the P1 group compared to the control group, although this enhancement was not statistically significant (Table II).

Overall, the P1 post-curing process showed high cyto-compatibility after 7 and 14 days, with a cell viability of $107.12\% \pm 17.47\%$ and $106.74\% \pm 18.41\%$, respectively. In contrast, the P2 procedures reported significantly moderate cytotoxicity ($P < 0.001$), with a mean percentage of surviving cells of $59.79\% \pm 10.06\%$ after 7 days, and $47.09\% \pm 20.62\%$ after 14 days (Table II).

Discussion

The purpose of the present study was to compare the cytotoxic levels of a 3D-print material used for

Table II. Descriptive and Inferential Statistics of Cell Viability, expressed in percentage (%)

Group	Day 7		Day 14	
	Mean $\pm$ SD (%)	Cytotoxicity	Mean $\pm$ SD (%)	Cytotoxicity
P1	107.12 ± 17.47 A ^a	None	106.74 ± 18.41 A	None
C+	100.00 ± 24.71 A	/	100.00 ± 18.61 A	/
P2	59.79 ± 10.06 *** B	Moderate	47.09 ± 20.62 *** B	Moderate

*** $P \leq 0.001$ indicate statistically significant differences compared with the controls.^a Different letter in the subgroups indicate the statistically significant differences among materials.

clear aligner manufacture under two different post-curing conditions. The null hypothesis that the post-curing procedures would not influence the relative cytotoxicity of 3D-print materials has been rejected.

As recommended by the manufacturers, post-polymerisation is necessary to avoid the initial toxicity of the 3D-print material.^{12,24} 3D-print resins are highly cytotoxic before the 3D-printing process, and the level of cytotoxicity is significantly reduced following the post-polymerisation procedures, which involve the removal of uncured resin.^{15,24}

However, the cytotoxicity of 3D-print materials may be affected by several parameters, including the material's composition, the printing conditions (the type and speed of the printer device, or the orientation of the aligner on the build plate), and post-processing procedures (washing and post-curing).²⁰ Therefore, each step in the material's processing stage may have a negative impact on its biocompatibility.²⁰

This aspect is of great significance as a wide range of 3D-printers and post-curing machines are commercially available and the manufacturer recommendations are not always followed by the end-users.²⁰

In the present study, Tera Harz TC-85 resin, a photopolymer material for the direct 3D-printing of aligners introduced by Korean Graphy Inc. company,¹² was used to manufacture several copies of 3D-printed aligners of the same size and geometry. To assess the overall cytotoxicity of the material under different post-curing procedures and minimise the impact of additional variables on the level of cytotoxicity, the aligners were printed using the same 3D-printer.

The polymerisation of photopolymer resin is a complex procedure, which depends, not only on the characteristics of the material but also on the properties of the curing machines.^{20,25} According to previous studies,²⁶ the degree of conversion of 3D-printed resin can be specifically enhanced by factors such as oxygen inhibition, high UV wavelength, the intensity of the curing light, and the temperature during the post-curing reaction.²⁷

Most 3D-print resins are cured by free radical polymerisation through UV irradiation. When the UV light wavelength is absorbed by a material, free radicals are produced which induce cross-linking of the oligomers and monomers within the resin, resulting in a polymer of cured material. However,

even using the correct amount of UV light, if the resin is exposed to the atmosphere during the curing process, oxygen may penetrate the top layer and inhibit complete surface polymerisation, resulting in an under-cured polymer with unreacted oligomers and monomers.²⁷ Therefore, minimising residual monomer content in 3D-printed aligners is necessary to reduce later contact with the oral tissues.²⁰

The Graphy company strongly recommends post-curing the aligners using an external UV curing device (THC 2 UV Curing System) with a nitrogen generator to guarantee sufficient final polymerisation.²⁸ This is an external light curing device operating with a UV LED, at a wavelength of 405 nm and an intensity of 200 W, plus with a generator of nitrogen for oxygen removal.

Using two types of post-curing processes and devices, the present study compared two different curing conditions. In addition to the THC 2, the Form Cure was also assessed as it uses a 405 nm light source with 13 multidirectional light-emitting diodes, in association with a heated cure chamber that can reach a temperature of 80°C.¹⁷

The P1 group showed high biocompatibility, while the P2 group exhibited moderate cytotoxicity. However, after 7 and 14 days, the aligner post-cured with THC-2 (P1) was not found to be cytotoxic, and a higher cell proliferation was observed compared to the control group. According to previous findings,^{20,29} this could be explained by the improved cell adhesion of 3D-printed materials, which exhibited a greater surface area for cell adhesion and may result in greater cell viability compared to controls.

The significant interaction between the post-curing procedures and the cytotoxicity of the aligners may be explained by the different technical properties of the two curing devices. Although both curing machines used a long wavelength to achieve better surface curing, two factors in the P1 group may have improved cell viability, resulting in high biocompatibility of the P1 resin. The increased light intensity of THC-2 and the possibility of curing under an inert gas like nitrogen to exclude oxygen from the environment resulted in an improved post-polymerisation process with a shorter exposure time (14 min).

However, even though the P2 post-curing conditions involved the use of a higher temperature and a

longer curing time, the P2 resin showed moderate cytotoxicity, likely due to the less powerful UV curing lamp and the lack of oxygen inhibition associated with the FormCure machine.

Therefore, due to the dependence of the cytotoxic properties of 3D-printed aligners on the post-curing procedures, the assessment of the optimal conditions for the post-polymerisation of a newly 3D-printed material is indicated, considering the wide range of post-curing conditions.

The current findings enabled a preliminary assessment of post-curing parameters affecting the cytotoxic effects of 3D-printed aligners.

The biocompatibility of dental materials is an important issue³⁰ because it indicates the ability of a material to interact with a biological system without causing injury³¹ related to toxicity or harm to the oral environment.³⁰ Testing the cytotoxic effects of dental materials using cell culture techniques is relatively simple, repeatable, cost-effective, and controllable. MC3T3E-1 mouse pre-osteoblasts have previously been useful for analysing cytotoxicity and provide an accurate assessment of a broad range of adaptive cellular responses.

Although this should be considered a limitation of the study, the applied tests were a more viable alternative to animal experiments, which may introduce uncontrolled variables.³² Further studies are recommended on human cellular cultures.

Conclusions

Different post-polymerisation procedures may affect the in-vitro cytotoxicity of 3D-print resin.

3D-printed aligners, post-cured using a Tera Harz Cure incorporating a nitrogen generator, were found to be biocompatible. In contrast, aligners post-cured using FormCure resulted in mild cytotoxicity.

Orthodontic laboratories and clinicians should follow the manufacturer's recommendations to avoid possible toxic effects during aligner treatment.

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Conflict of interest

Marco Migliorati is involved in Graphy's educational events with Yen Co., Italy (one of the Italian Graphy resin resellers). The other authors declare that there is no conflict of interest.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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