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BIOCOMPATIBILITY OF 3D PRINTED PHOTOINITIATED PROSTHODONTIC RESINS (In vitro Study)

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ABSTRACT

INTRODUCTION: The production of numerous types of materials for diverse uses in dentistry is a rapidly growing and research-intensive field. Although 3D printed resins are among the many materials utilized in prosthetic appliances today, research on their biocompatibility in long-term dental applications is limited.
OBJECTIVE: Evaluation of cellular behavior of human gingival fibroblasts "HGFs" when put in contact with 3D photoinitiated printed resins and heat polymerized acrylic resin.
METHODOLOGY: HGFs were isolated from attached keratinized gingival tissues from healthy patients who had crown lengthening procedures, cellular viability using MTT assay in response to 3D printed resin Dental LT Clear (FORM LABS), compared to Heat-polymerized acrylic resin (Acrostone)which was assessed throughout four-time intervals (24, 48, 72 and 168 hours).
RESULTS: All groups' cell viability decreased over time, although with favorable cell viability of more than 90% and non-cytotoxicity. It was found to be significant among Heat polymerized acrylic resin (RG), while among Dental LT Clear (TG) the decrease in cell viability were statistically insignificant. The highest cell viability was found after 24 hours among all groups; however, the least viability was found after 48 hours among RG, and among TG after 72 hours. There was a non-statistical significant difference in cell viability after 168 hours.
CONCLUSION: Throughout time intervals and for long term use, it was found that photoinitiated resin were less cytotoxic than heat polymerized acrylic resin but with no statistically significant difference.
KEY WORDS: Biocompatibility, 3D printed photoinitiated resins, CAD/CAM, Cytotoxicity

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INTRODUCTION

The efficacy of various resins used in computer aided design/computer aided manufacturing (CAD/CAM) dental treatments is based on their physical, chemical, and biological properties. Dental materials' biocompatibility is an important consideration in their clinical application (1–3). Biocompatibility describes the ability of a material to perform with an appropriate host response when applied as intended (4).

The release of chemicals through solubility or corrosion is a major determinant of a material's biocompatibility. By promoting the synthesis of particular proteins in cells, these chemicals might degrade them or cause inflammation. Toxicity refers to a material's ability to cause damage to biological tissues, which can range from poor metabolic activity to organ damage and cell death (5). Biocompatibility includes cytotoxicity as a key component (6).

In the twenty-first era, CAD/CAM technology has emerged as one of the most significant advancements in the dental industry (7). Early CAD/CAM systems only relied on subtractive 'milling' technique. (8). A rapidly growing alternative to milling is known as Additive manufacturing (AM) printing (9). The term three-dimensional (3D) printing refers to the technique of connecting materials to create items using 3D model data, which is commonly done layer by layer (10). Recently, additive technology has outperformed other digital manufacturing processes due to the flexibility of employing a numerous materials and machines, as well as the technology's passive character. Furthermore, there is a low percentage of lost raw material, as 3D printing machines waste 40% less than subtractive machines, and roughly 95% to 98 % of waste may be recycled. (10,11).

AM's diverse materials and techniques have resulted in a number of dental applications, including implant surgical guide templates and occlusal stents for orthognathic surgeries (12,13), as well as fabrication of interim dental restorations (8,14)**.** Also, it can manufacture other appliances which are intended for extended time of use such as interim obturators for maxillary defects (15), sleep apnea devices (16), removable partial dentures metal framework, complete dentures and implant supported fixed dental prostheses (8).

Biocompatibility of materials used in traditional and subtractive dental materials are clearly documented; however, the influence of resins used in Stereolithography (SLA) is presently unclear. (17–19). However, due to residual monomer and photo-initiator, most photosensitive resin materials are cytotoxic, affecting cell survival and physiological activities since monomer diffusion impairs gingival cell viability (20–22). Thus, even if a resin has already been declared biocompatible, commercial resins must be evaluated for biocompatibility for each use separately (23). Academic research on photocuring 3D printing materials for direct and long-term interaction with living bodies is still ongoing (20)**.**

These considerations and limitations of studies about the biocompatibility and cellular effects of 3D printed resins were the motivation to carry out the present study which aimed to assess the cytotoxicity of photoinitiated 3D printed resin compared to the heat-polymerized acrylic resin. The Null hypothesis is that 3D printed resins is biocompatible and will not show higher cytotoxic effect compared to heat polymerized acrylic resins.

MATERIALS AND METHODS

Sample size estimation

Sample size was based on 5% alpha error, 80% power and a standardized effect size of 0.9215 derived from a previous study (24). The minimum sample size was 8 discs per group (two groups) at each time interval (four-time intervals), giving a total sample of 64 resin discs.

Study design

For this study, 32 3D printed photoinitiated resin disc samples and 32 heat polymerized acrylic resin samples, were used to evaluate their cytotoxic effect on HGFs. Heat-polymerized acrylic resin was used as comparing group to compare the cytotoxic effect of the printed resin to its effect on oral tissue. All samples were assessed for their cytotoxic effect on HGFs, that were extracted from healthy attached gingiva then assessed after 24, 48, 72 and 168 hours (7 days).

Fig.1: Dental LT Clear resin discs printed by FormLabs Form 2 machine

Preparation and Manufacturing of 3D samples

The 3D samples of test groups (TG) were designed, and 3D printed using CAD software (ExocadGMBH, Germany) with a diameter of 30 mm and a thickness of 4 mm, then translated to a Standard Tessellation Language file " STL file". STL file was transferred to the printer using custom machine software (25). All 3D printing was performed via SLA. The Dental LT Clear resin (FLDLCL01) was printed by a FormLabs Form 2 machine (Formlabs, Somerville, MA, USA) (Fig.1).

Materialise MiniMagics was used to verify STL files. At a wavelength of 405 nm, printing parameters were set to 50 mm s1, 0.1 mm layer height, and 100 % fill density. Using a knife, the samples were then removed from the platform. The printed samples were then rinsed twice in an ultrasonic bath (CD-4820 Codyson, Misr Sinai, Egypt) filled with pure isopropyl alcohol (IPA) (El Nasr Pharmaceutical Chemicals Co., Egypt) to remove any excess material, rinsed the first time for 15 minutes, then discs were removed and soaked again in fresh IPA for an additional 5 minutes before post-curing. Then the support structures were removed using a cutter. After cleaning and drying, the printed samples were placed in an ultraviolet (UV) light curing box for 20 minutes on each side for final polymerization to guarantee that the printed samples obtained full polymer conversion, minimal residual monomer, and the optimum mechanical properties. This method was optimized to obtain the manufacturer's goal of a biocompatible end product (26).

Fabrication of resin group samples

Heat polymerized acrylic resin samples (RG) (Acrostone, Heliopolis, Cairo, Egypt.) were fabricated by creating a print space in stone molds within a dental flask, by placing previously finished 3D samples in the mold, then packing and processing according to the manufacturer's instructions. Finally, as with an actual acrylic resin denture base, the samples were finished and polished (1).

Both 3D printed and heat polymerized acrylic resin samples were disinfected for 5 minutes with 70% ethanol (October Pharma S.A.E., Egypt), then rinsed with phosphate buffer saline (PBS) (Biowest, Business Park Lane, USA). To prevent bacterial contamination, all samples were placed separately in sealed sterilization pouches and sterilized under UV radiation for 60 minutes in a biosafety cabinet (EscoMicroPte.Ltd, Singapore) before each test (24).

Tests were conducted to determine the resin's indirect effects after 24, 48, 72, and 168 hours (7 days) intervals in growth media (GM) through their cellular viability among the corresponding time intervals.

Samples were placed in six well plates of culture area that were acellular and contained MEDIA ONLY to evaluate the indirect cellular response to the chemical leachate from each resin. During each repeat, each well was used to transfer chemical leachate medium to its corresponding experimental well, ensuring that the transferred medium accurately reflects cumulative resin degradation or control at each time-point (fig.2). The conditioned media were stored at -20 ^{0}C till the commencement of the cytotoxicity study.

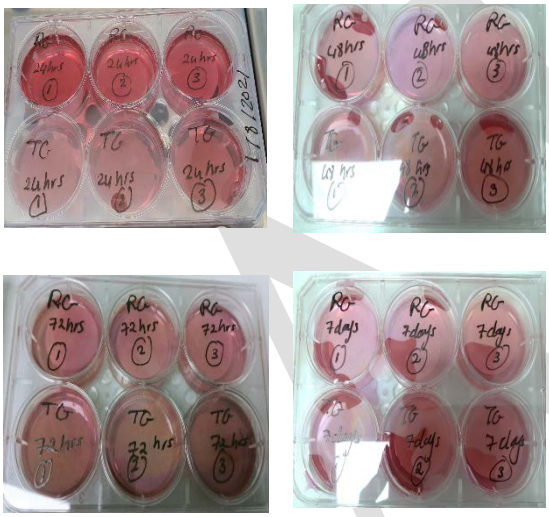

Fig.2: Study groups samples in six well plates containing a cellular culture media

Cell culture preparation and isolation

Attached keratinized gingival tissues were obtained from healthy patients undergoing crown lengthening operation. A protocol authorized by the Ethical Committee of the Faculty of Dentistry, Alexandria University, Egypt, was used to gain signed informed consent from the donors.

A sample of keratinized gingival tissue was collected from donors under local anesthesia then transported to the laboratory in a sterile falcon tube containing PBS + 3% penicillin/streptomycin/amphotericin (containing 10,000 IU/mL penicillin; 10,000 g/mL streptomycin; and 25 g/mL amphotericin B, Lonza), then de-epithelialized. The gingival samples were then washed three times in PBS before being cut into 1x1 mm fragments. Fragments were then maintained in low-glucose tissue culture dishes (Dulbecco's modified Eagle's medium (LG-DMEM) supplemented with 10% fetal bovine serum, 2 mm L-glutamine, 1% penicillin/streptomycin (Biowest, Business Park Lane, USA) (Fig.3) and left in incubator with 5% CO_2 at 37 ^{0}C (27).

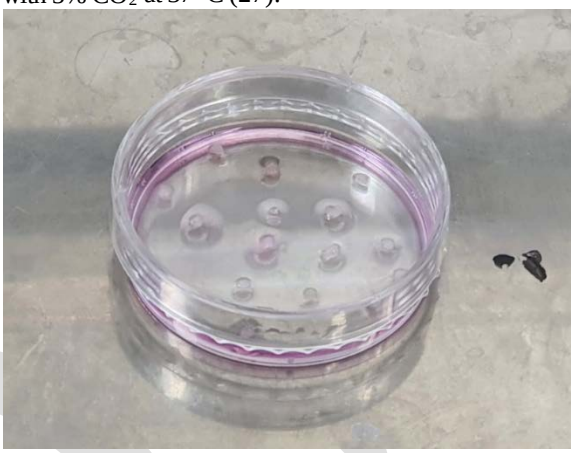

Fig.3: Culturing the Tissue fragments in tissue culture dishes

GM was renewed every 2 to 3 days for a total of 14 days to allow the tissue explanted fibroblasts to attain 80%–85% confluence. The cells were detached from the monolayer using trypsin EDTA (0.25 % trypsin, 1 mM EDTA) (Biowest, Business Park Lane, USA) and sub cultured in tissue flasks under the same conditions until passage 4 was attained (Fig.4) (24).

MTT assay

The cell viability of HGFs was assessed by an MTT [(3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test, it shows how changes in mitochondrial dehydrogenase activities affect cell viability. It is based on the water-soluble methylthiazole tetrazolium being converted to an insoluble purple formazan (28).

Cells at passage 4 were planted at a density of $7x10^3$ cells per well in 96 well plates and cultured for 24 hours to become adherent and around 70% confluent. After that, the plates were split into three groups: Control group (CG), Resin group (RG), and Test group (TG). Control group (CG) cells were cultured in Complete growth media to be used in calculating the cell viability percentage, Resin group (RG) cells were cultured in Heat polymerized acrylic conditioned media, and Test group (TG) cells were cultured in Dental LT Clear conditioned media (Fig.4). GM or conditioned media collected through the time intervals

from all resins were transferred to the cells and incubated for 24 , 48 , 72 hours, and 7days.

Table 1: Cell viability at different time intervals among the study groups

	RG (n=8)	**TG (n=8)**	**t Test (*p* value)**
	Mean (SD)		
24 hours	99.86 (3.39)	99.64 (4.29)	0.115 (0.910)
48 hours	87.62 (4.41)	97.12 (6.22)	**3.523 (0.003*)**
72 hours	92.09 (3.73)	93.20 (6.63)	0.410 (0.688)
168 hours	92.13 (2.07)	96.71 (7.06)	1.757 (0.101)
Repeated Measures ANOVA Test (*P* value)	22.079 **(<0.0001*)**	1.848 (0.216)	

*Statistically significant different at *p* value ≤0.05
RG=Resin group (Heat-polymerized acrylic resin)
TG= Test group (Dental LT Clear)

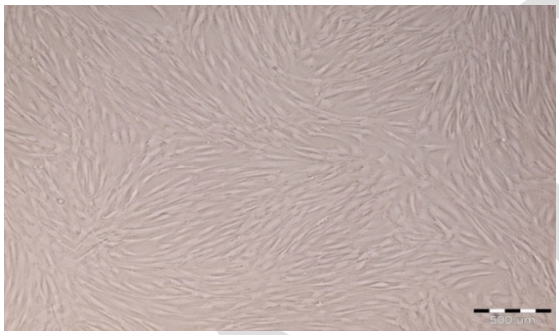

Fig. 4: Explanted fibroblasts after reaching passage 4
After incubation, the cells were rinsed in PBS and the media replaced with 0.5 mg/mL MTT (Trevigen, Helgerman CT, Gaithersburg, MD, USA) in DMEM for 3 to 4 hours at 37 °C. The formed crystals were dissolved in Dimethyl Sulfoxide solvent solution (fig.5), and the optical density was measured at 570 nm. An UV reader (Tecan Trading AG, Switzerland). was used for all of the readings Three separate MTT assays were carried out, with eight replicate wells for each experimental point. All the steps were done in the biosafety cabinet class II to prevent contamination and to protect the operator and samples

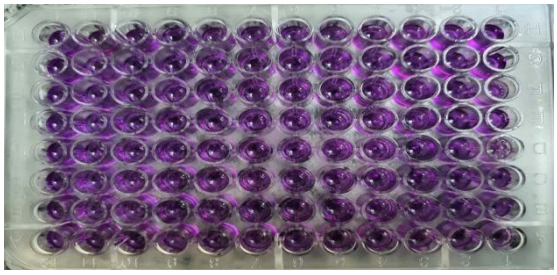

Fig.5: HGFs viability assessment using MTT test

The viability percentage was calculated using the formula: Cell viability (%) = (optical density of the test group ÷ optical density of cellular control group) x 100.

Statistical analysis

Normality was checked using Shapiro Wilk test, box plots and descriptives. Data was normally distributed and presented using Mean and Standard deviation (SD).

Groups' cell viability was compared using independent t test. A change across time was assessed within each group using Repeated Measures ANOVA test. Significance level was set at p value of 0.05. All tests were two tailed. Data were analyzed using SPSS for windows version 23

RESULTS

The cell viability percentage of study groups material was examined across time periods of 24, 48, 72, and 168 hours in contrast to the control (MEDIA ONLY). There was a favorable decrease in cell viability of all groups throughout time intervals, which
was found to be greater than 90%, indicating that all utilized resins were not cytotoxic (Table 1).

According to independent t test, statistically significant difference was only found among study groups after 48 hours with p value of 0.003, where RG showed the least cell viability (87.62%) and TG showed higher cell viability (97.12%). While through time intervals (Repeated Measures ANOVA), the decrease in cell viability was found to be statistically significant among RG only (P = <0.0001) (Table 1).
In addition, the results showed that the highest cell viability was found after 24 hours among all groups; however, the least viability was found after 48 hours among RG and among TG after 72 hours (Graph 1).

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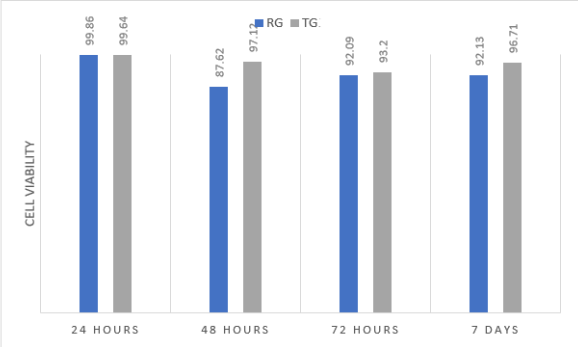

Graph 1: Mean cell viability at different time intervals among the study groups

When the cell viability percentages of photoinitiated resins and heat polymerized acrylic resin were compared over time intervals for long-term use, it was discovered that photoinitiated resins were less cytotoxic than heat polymerized acrylic resin, but the difference was not statistically significan

DISCUSSION

Resin-based materials have been found to generate adverse effects in the oral mucosa, so their biological behavior needs to be assessed and evaluated (29).

The in vitro test was used to investigate the biological impact of resin-based materials on oral mucosa. It was chosen because it has been demonstrated to be reproducible, experimentally controllable, rapid and relatively easy, affordable, and free of ethical concerns (30). The MTT assay was employed to determine the vitality of cells exposed to test materials since it is the most frequently utilized test because it is a simple and inexpensive method, as many authors have demonstrated (1,2,21,31,32).

In the present study, the HGFs were selected for viability assessment as they are dominant resident cells in gingival connective tissue, quickly growing in standard culture medium, and have a high sensitivity in cytotoxicity assays. (30,31).

Since sufficient contact between cells and evaluated material is critical in the biological evaluation (33), a modified technique of cell contact through extracts and elutes method (indirect) was used in the current investigation. To keep the cells in contact with the elutes and provide more accurate cell response, the cells were cultured in conditioned media for the same amount of time as the resin was left in the growth media.

In this study, the cytotoxic effect of Dental LT Clear 3D printed photopolymer resin, which is ideal for hard splints, occlusal guards, and other direct printed long term orthodontic appliances, with LT standing for long term FDA Class IIa biocompatibility [17,33,38,40,41], was assessed on HGFs and compared to conventional heat polymerized acrylic resin material, as it is the traditionally preferred and used resin [31,35–37].

The results of this study revealed that, all materials found to have non-significant cytotoxic effect on HGFs with a visible trend of an insignificant decrease in cell vitality from 24 hours to 168 hours. All resins had the least cytotoxic effect on HGFs after 24 hours, while highest cytotoxic effect found to be after 48 hours among heat polymerized acrylic resin, these findings are supported by Beiger et al's study, which used high-performance liquid chromatography to assess monomer release from conventional dental resins into saliva, and found that the maximum release occurred within the first 24 to 48 hours (34). while for Dental LT resin the highest cytotoxic effect was after 72 hours but without significant value. After 168 hours (long term interval) there was slight reduction in cell viability and consequently slight increase in cell cytotoxicity. The least cytotoxic effect was found among Dental LT. The results of some cytotoxic effect of dental resins may be attributed to their content of polymethylmethacrylate which was previously reported to be the reason of resin cytotoxicity as found by Fayyaz et al (21) who reported that polymethylmethacrylates are present in photoinitiated resins, and the release of methacrylate monomer may be the cause of the resins' cytotoxicity.

Although Dental LT Clear photoinitiated 3D resin has previously been classified as biocompatible, the discrepancies with the current study's finding of slight cytotoxicity could be due to different incubation periods for cytotoxicity testing; where the longer incubation period used in the study could lead to gradual long-term effect accumulation, implying that a longer incubation period could lead to even lower cellular viability, as reported by Kreb et al (23), that support the results of the present of reduced cell viability through time intervals. Moreno et al. (35), who evaluated the biocompatibility of Dental LT resin, discovered that the biocompatibility test revealed a decline in the number of viable cells across time intervals, which could be attributed to a delayed release of chemicals. These findings back with our findings of reduced cell viability linked to methylmethacrylate release.

While at 2020, Fayyaz et al. [17] employed the MTT assay to assess the in vitro cytotoxicity of the direct printed aligner utilizing Dental LT clear resin on 3T3 mice fibroblast cells at various time intervals, where the extraction medium was changed on the first, third, fifth, and seventh days. The authors noticed a considerable improvement in cell viability from day one to day seven. The fact that only one sample of the

used resin was used, and the medium was changed and examined at each time interval, could explain the discrepancy in our results. Another aspect that could have affected the outcome was the cell line that was employed.

Regarding to heat polymerized acrylic resin, Çakırbay et al. [31] investigated the cytotoxicity of heat polymerized resins and come to the same conclusion as the current study. The samples were kept in water for either 24 hours or 15 days. After 72 hours of cell incubation, cytotoxicity was assessed using the MTT test using L929 cells. After 24 hours of water storage, cell viability was observed to be high, but then dropped after 15 days.

The null hypothesis was accepted based on the results of the experiment, as the study revealed that there were no statistically significant differences in HGFs cytocompatibility between photoinitiated resin and heat polymerized acrylic resin. Despite the decrease in cell viability of all specimens, but they still falling in accepted cytotoxicity level except some of heat polymerized acrylic resin specimens.

Given the research's limitations, in vitro test methods have several drawbacks, including a lack of direct association with clinical conditions, the inability to culture cells for more than 7 days, and the use of only one type of cell line. Further experimental and clinical tests must be conducted.

CONCLUSION

When compared to heat polymerized acrylic resin, the 3D photoinitiated resin was found to be more biocompatible. Cytotoxicity rises over time, implying that the slow release of chemicals with longer incubation intervals will have a long-term effect on diminishing cellular viability, but only to a level that is acceptable. Within the research's limits, 3D printed photoinitiated resins are advised for long term use.

CONFLICT OF INTREST

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