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Cytotoxic evaluation of directly 3D printed aligners and Invisalign

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Abstract: Background: Direct 3D printing of aligner trays involve printable materials; the study aims to investigate the in-vitro cytotoxicity of the direct-printed aligner using photopolymer resins and SmartTrackInvisalign tray for varying time intervals on 3T3 mice fibroblast cells using MTT assay.

Materials and Methods: Directed printed aligner trays using two 3D printing materials with SmartTrackInvisalign tray were compared in this study. Samples were placed in Dulbecco's Modified Eagle's Medium (DMEM; 0.1 mg/mL) for 1,3,5& 7 days interval. Cell viability percentage was calculated, and data were analyzed using a one-way analysis of variance and post hoc tests (α = 0.05).

Results: All materials exhibited slight cytotoxicity on MFCs with a visible trend of a significant increase in cell viability from day 1 to 7. Among the groups, the higher cytotoxicity was by E-Guard clear, and Dental LT, and the least cytotoxicity by Smartrack material. The highest level of cell viability and no cytotoxicity was exhibited by Invisalign (94.07% ± 3.00 of cell viability) at day 7. No statistically significant difference in viability percentage was seen between Dental LT and E-Guard material.

Conclusions: SmartTrackInvisalign material (polyurethane) was found to be more biocompatible, followed by directly printed aligner materials (polymethylmethacrylate). Cytotoxicity was found to be more on the first day for all materials and gradually decreases as day's progress. The results indicate the increased leaching of material during the initial period of use though the level of cytotoxicity is slight.

Keywords: 3D printing; Directly printed aligners; Biocompatibility; Cytotoxicity; MTT assay

1. INTRODUCTION

Digital workflow in orthodontics has gained popularity as they enable faster work processes and treatment. Such workflow involves acquiring three-dimensional (3D) data, virtual modeling of the digital model, or digitally designed appliance using acquired data and physical printing with 3D printers. The next paradigm shift in orthodontics happened with the development of 3D printers, working in conjunction with intraoral scanners [1]. This workflow can be dental lab dependant if the process involves the lab to fabricate the end-use product like aligners or can be independent of lab production with in-office aligner fabrication using a 3D printer. Aligners and digital models printed with a 3D printer offer a diverse application inpatient management [2,3].

3D printing presently stands as the dominant technology for orthodontic model production in clear aligner therapy, due in part to the decreasing cost of 3D printers. The importance of thematerial applied in 3D printing plays a pivotal role in the workflow for aligner fabrication[4-6]. The optimal printing parameters for a given material typically require the use of specific printers capable of achieving those conditions, so the material selected must be compatible with the format, make, and model of the 3D printer to be used. Otherwise, the anticipated physical properties, such as mechanical strength, of the printed material, might not be achieved. Additionally, potential effects of the materials themselves on patients and personnel should be taken into consideration, as residual resin monomer might present toxicity to patients if transferred with the appliance, and patients and staff may develop allergies to acrylics with exposure [3-7].

Today, 3D printing material used in appliance fabrication differs based on the components printed, its application, and biocompatibility. Digitally printed appliance for intra-oral usage needs to be under biocompatibility standards. Photopolymer resins used in 3D printing for intraoral applications are categorized as short-term (<24 hours) or long-term usage (>72 hours) [8]. Directly printed aligners warrants long-term biocompatibility as the appliance wear is for 14 days with a daily wearing time of 20-22 hrs[9,10]. Digitally printed surgical guides, mini implants placement templates, debonding bite wafers, and indirect bonding trays use materialsof short-term biocompatibility.

Thepreferable material for orthodontic 3D printing of models is polymethylmethacrylate resin (PMMA)[11]. Recently, stereolithographic printed Dental LT resin (Formlabsinc.) and digital light processing (DLP) printed E-Guard clear resin (EvisionTec) are used for rapid prototyping of intra-oral CAD appliances likeretainers, aligner trays, and mouthguard. These photopolymer resins offer long termbiocompatibility. Studies were done on accuracy and, mechanical properties of 3D printable photopolymer resins suggest using these resins would provide the benefit of direct 3D printed appliances, especially aligner trays eliminating the process of digital model printing followed by thermoforming the aligner trays [9-15].

Since 1999, Invisalign® (Align Technology, Santa Clara, California) uses thermoformed polyurethane for its aligner fabrication combined with stereolithographic 3D printed model. Studies done to evaluate the cytotoxicity of polyurethane aligners (Invisalign®) concluded the material to be safe for intraoral usage [16-18].Recent recommendation for aligner usage with a maximum aligner tray and one week change compared to the previous 14 days, reduces the wear and tear of the aligner and improves compliance [2]. Most of the 3D printable materials are resin and Bisphenol-A, one of the by-products resulting from degradation of such resins, can act as a steroid hormone and cause biological effects, such as premature puberty in girls, ovarian cancer, or disruptive maturation of male reproductive organs. However, some investigators believe that the quantity of bisphenol-A released from these materials is lower than that required to induce a biologic reaction [17]. Invisalign has made time to time improvement with their aligner tray material over the years, and SmartTrack® material is the recent offering to meet the Food and Drug Administration (FDA) requirement in treating more complex malocclusion with long term usage [2].

Cytotoxicity assay techniques like Tetrazolium reduction assay has been widely adopted to investigate material safety. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction assay is a colorimetric assay based on assessing the cell metabolic activity to quantify cell death and cytotoxicity [19,20]. Viable cells with active metabolism convert MTT into a purple coloured formazan product. When cells die, they lose the ability to convert MTT into formazan. Thus, colour formation serves as a useful and convenient marker of only the viable cells and assesses the cytotoxicity. Cell viability percentage is inversely proportional to cytotoxicity, and as higher the viability percentage lesser is the cytotoxicity.

As the printing technology evolves, so are the 3D printing materials, studies should assess their biocompatibility for safer intra-oral usage. In this article, we evaluated the cytotoxic effects of 3D printedphotopolymer resins used in the rapid prototyping of direct-printed aligner trays compared to thermoformed SmartTrackInvisalign® trayfor its epithelial cell response through cell death usingthe in-vitro assay.

2. AIM AND OBJECTIVES

This present study aims to evaluate the cytotoxicity of directly 3D printed and thermoformed aligners. The in-vitro cell viability was evaluated onSmartTrackInvisalign® material as the thermoformed aligner and direct-printed aligner using newer long-term 3D printing photopolymer resin materials for varying time intervals using MTT assay and its biocompatibility for intra-oral usage.

3. MATERIALS AND METHODS

This in-vitro prospective cytotoxicity study was conducted in 3D printed aligner trays made of Dental LT and E-Guard clear materials and thermoformed SmartTrackInvisalign aligner. The study involved various steps in evaluating cytotoxicity, which is as follows 1) sample preparation, 2) cell culture, 3) cytotoxicity assay, and 4) Cell viability percentage.

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Sample Preparation

Scanned Impression of the patient dentition was used to design an aligner tray using 3D modeling software. The direct 3D printed aligner tray was designed using the point plotting method to eliminate the aligner tray extending over the gingiva. For the aligner tray printed with Dental LT, a thickness of 0.75mm was fixed and printed using stereolithographic technology in Form 2 3D printer (Somerville, Mass). After 3D printing, the aligner tray was washed with 96% isopropyl alcohol and post cured as per the manufacturer recommendation with Form Cure unit were a 405nm light is used to cure at 80º C for 20 minutes.

A similarlydesigned aligner tray was used for 3D printing with E-Guard clear material (EnvisionTEC, Rockhill, South Carolina). Digital light processing technology (DLP) using the EnvisionTEC VIDA HD printer (EnvisionTEC, Rockhill, South Carolina) printed the tray for 25 minutes with 0.75mm thickness followed by rinsing at strong-mode with PWA 2000 (EnvisionTEC, Rockhill, South Carolina) parts washing apparatus. Post curing was done using UV Curing Apparatus (UVCA 2000) for a total of 5 minutes (Flipping the tray at half time) to cure any uncured resin during DLP and increase wear resistance based on manufacturers instruction.

For the SmartTrack® (San Jose, California) aligner tray, a lower arch refinement tray of a patient was used as the sample. As SmartTrack® tray is polyurethane plastic, it was considered as a thermoformed material instead of direct 3D printed material in this study for cytotoxic evaluation. The surface area covered by three trays were measured using a graph paper according to international standards organization (ISO 10993-5 and 10993-12) for assessing the cytotoxicity of medical devices [21,22]. The surface area measurement was done to quantify the amount of extraction medium needed for each of these samples, as in Figure 1. Splints made of E-Guard and Dental LT resin-covered 11.96cm^2 surface area and required a 2ml extraction medium. Invisalign covered 8.81cm^2 surface area and need 1.5 ml of extraction medium. After measuring, the surface area covered and medium of extraction required, samples were sterilized following the protocol defined by the ISO norms [21].All three samples were then kept in a 100mm petri dish (Corning®). Dulbecco modified Eagle medium (DMEM) (Gibco®, Invitrogen) served as the extraction medium for this study.

The extraction medium was changed at 1st, 3rd, 5th, and 7th day (Figure 2). After each time interval, the culture medium was removed, and a new culture medium was introduced into the samples. The removed culture medium was then labelled for each time interval (i.e., 1^{st}, 3^{rd}, 5^{th}, and 7^{th} day) and stored at -20 degrees Celsius until the commencement of the cytotoxicity study using MTT assay on the 8^{th} day.

Cell line culture

Mouse embryonic fibroblast cell lines - 3T3 mice fibroblasts(MFCs)were obtained from the National Centre for Cell Science, Pune, India. The mouse fibroblast cell linewas cultured in Dulbecco Modified Eagle medium (DMEM) with 5% fetal calf serum (Himedia), penicillin, and streptomycin. The cell line and culture medium incubated at 37°C in an atmosphere of 95% air and 5% CO_2. Once the cells attain 80% confluence, these cells were transferred to 91

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wells of 96 well tissue culture grade plate (Corning®). Nearly 5,000 cells seeded per plate in 91 wells along with standard cell culture medium (DMEM).

Cytotoxicity assay

The mice fibroblasts were plated on 91 wells of 96 well microplate (5000 cells/well). The culture medium (DMEM) was then removed from these cells and replaced with the stored culture medium (100 µl/ well). The microplate was divided for each sample group, i.e., 28 wells (7 wells each for 1st, 3rd, 5th, and 7th day) forSmartTrack®, 28 wells for Dental LT, and 28 wells for E-Guard and 7 wells serve as control were in cells grow in a normal culture medium.

After 24-hour incubation of 91 wells with the plate, MTT assay was done using the MTT assay kit (Merck®). MTT tetrazolium solution of 5µl per well was added to the cells and incubated for 5 hours at 37^0C. At the end of the incubation period, the dye was removed and 100 µl of DMSO (Dimethyl Sulfoxide) was added to the wells as in Figure 3. Finally, the optical density was measured in an ELISA plate reader (Biotek technologies) at 540 nm. Cell viability of these MFCs was assessed as cell viability percentage using the following formula, and the results were tabulated for statistical analysis.

Cell viability calculation

The viability percentage was calculated using the formula: Cell viability (%) = (optical density of the test group ÷ optical density of cellular control group) x 100.Cell viability was scored according to the classification of Ahrari et al. were >90% cell viability is no cytotoxicity, 60%-90% cell viability is slight cytotoxicity, 30%-59% cell viability is moderate cytotoxicity and <30% cell viability indicatessevere cytotoxicity [23].

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 20.0, SPSS IBM, Armonk, NY, USA). Differences between mean values determined by one-way analysis of variance (ANOVA) with Bonferroni post-hoc test. The level of significance was set at P-Value 0.05.

4. RESULTS

Cell viability of the materials with their mean value and standard deviation are shown in Table 1 and the corresponding level of cytotoxicity in Figure 4. All materials exhibited slight cytotoxicity using the MTT assay on MFCs with a visible trend of significantly increasing in cell viability from day 1 to 7. Among the groups, the higher cytotoxicity was by E-Guard clear, Dental LT, and the least cytotoxicity by SmartrackInvisalign material. The highest level of cell viability and no cytotoxicity was exhibited by Invisalign (94.07% ± 3.00 of cell viability), followed by slight cytotoxicity of Dental LT (77.74% ± 3.22 of cell viability) and E-Guard (75.06% ± 8.98 of cell viability) at day 7. A statistically significant difference in cell viability was present between Invisalign and Dental LT, Invisalign, and E-Guard material. There was no significant difference in cell viability between Dental LT and E-Guard material.

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5. DISCUSSION

Ever-growing emphasis on esthetics and new technologies had led to the introduction of various esthetic alternatives to treating malocclusion. Malocclusion correction with aligners has gained immense popularity in recent times. Invisalign produced from stereolithographic models uses clear aligners made of polyurethane, which have been in use for over the past two decades in treating malocclusion [2,16-18].Increasing demand for newer aligner materials to eliminate the thermoforming process with direct 3D printing materials has gained momentum as it has benefits of being environment-friendly and faster workflow [9,10]. Photopolymer resin-like Dental LT, were LT stands for long-term FDA Class IIa biocompatibility, and E-Guard is another photopolymer resin with biocompatible standards commonly used to print retainer and mouthguard were used for direct aligner printing [9-13].

Recent studies suggested that printed aligners with offset using newer 3D materials offer the possibility of direct-printed aligners [9,10,13]. There are already a few studies carried out to check the cytotoxicity of thermoformed aligner trays [16-18].Those studies had varying results, from no cytotoxicity to slight cytotoxicity. No previous studies have assessed the cytotoxicity of direct 3D printed aligners. With interest in assessing the biocompatibility of newer 3D materials for long-term intraoral usage, this study was undertaken. In this prospective study, cytotoxicity of 3D printed aligner trays along with Invisalign was evaluated, as it will open newer possibilities for photopolymer resin usage in digital orthodontics. Direct-printed aligners eventually eliminate the printing of 3D printed models followed by the thermoforming process to fabricate aligner trays.

Various cell characteristics and functions are used to investigate the cytotoxicity of medical devices. In this study,a 3T3 embryonic mouse fibroblast cell line, which has similarities in cell lineage to human fibroblast, was used for studying cell viability.MTT assay is the most preferred and readily available cell line for cytotoxicity assay of medical instruments, equipment, and drugs. MTT is a tetrazolium Bromide reduction assay, and its mechanism is, healthy, viable cells with active metabolism convert MTT into a purple coloured formazan product with an absorbance maximum near 570 nm. The formazan crystals precipitate in the cell culture medium solution, and it must be dissolved before measuring optical density. For this purpose, dimethyl sulfoxide (DMSO) was used, which dissolves the water-insoluble formazan crystals to form a purple coloured liquid. More significant the change in colour, the higher is the proportion of healthy cells [16,18].

In our study, day 1 showed the maximum cytotoxicity level for Invisalign, and 3D printed materials as days progressed at day 7, the toxicity level gradually reduced. Intragroup findings of cell viability between the groups showed all materials had slight cytotoxicity, which is statistically significant (Table 1). According to Kopperud et al.**,** the cytotoxic effect is due to the genotoxicity of methacrylate monomer, whereby it directly affects the DNA by the formation of reactive oxygen species [24].Photopolymer resins are polymethylmethacrylates. The release of methacrylate monomer might be the reason for the slight cytotoxicity of directly printed aligners. These results were similar or lower thanthe cytotoxicity level achieved by many other dentalmaterials such as thermoformed aligners,

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orthodontic acrylics, metallic brackets, and bands,miniscrews, or bonding materials [18,19].There was no significant difference in cell viability between Dental LT and E-Guard material.

In this study, the cell viability was assessed only for a shorter period at 1st,3rd,5th, and 7th day. Recent recommendation for aligner fabrication with the maximum number of tray change is up to 7 days compared to the previous 14 days. Cytotoxic evaluation for shorter duration does not hamper the quality of this study as many studies have shown that cytotoxicity is more in the material during the first few days of intraoral usage[16-18].Long term studies that have evaluated the cytotoxicity have concluded that changes in cytotoxicity were severe in the first few days, and there was no significant increase in cell viability after the first one week of evaluation [19].

Previous studies suggested that polyurethane is a polymer of 4,4l di-methyl diisocyanate, and leaching of this causes cytotoxicity. Still, saliva acts as a buffer from the cytotoxic effects of isocyanate from the Invisalign tray [17,18]. In our study, among the groups, all had a statistically significant viability percentage. When compared to 3D printed materials, the Invisalign had increased cell viability and least cytotoxicity. In the Invisalign group, significant value to be noted was in day 7 as it exhibited no cytotoxicity compared to other time intervals and other groups. Both the Dental LT and E-Guard material had slight toxicity with no statistical difference among cell viability between them.

3D printed materials are highly toxic before 3D printing, and the toxicity gradually decreases post-polymerization. Post curing and processing are vital for eliminating the toxicity levels as recommended by the manufacture of 3D printing material. Post curing removes any uncured resin and makes the printed material much safer for intraoral usage. UV curing apparatus and subsequent washing are the recommended protocol for eliminating uncured resins to increase the mechanical properties like wear resistance of the end product and reduce cytotoxicity [9,11,13].

Direct printing of clear aligners could enable new horizons in aligner mechanics, by enabling spatial control of aligner properties, such as thickness, which is not feasible with current thermoforming methods [9,10].Dental LT and E-Guard photopolymer resin aligners showed slight cytotoxicity, which is within the norms of most of the thermoformed trays used for aligner fabrication [18].At the pace of orthodontic appliance customization, the recent improvements in 3D printers and human creative capacity, Orthodontists will be able to manufacture their aligners and, at each appointment, predict the movement they produce. Both material and host characteristics influencethe biocompatibility phenomena, and therefore, the results summarized in this studyshould be carefully analyzed for clinical application. We suggest future studies to evaluate the new EnvisionTEC material for the direct 3D printing of aligners, theE-Orthoign® material, for its cell viability. We also suggest evaluating the role ofdifferent cytotoxicity assays and their results in future orthodontic research.

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6. CONCLUSION

- SmartTrackmaterial (polyurethane) found to be more biocompatible, followed by direct-printed (polymethylmethacrylate) photopolymer resin aligner trays.
- Cytotoxicity was found to be higher on the 1st day and gradually decreases as days progress, suggesting increased leaching of material during the initial period of use.
- Dental LT and E-Guard clear, direct 3D printed aligners exhibit slight cytotoxicity of acceptable range compared to the thermoformed aligner.

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Table 1

	Invisalign (A)		**Dental LT (B)**		**E Guard (C)**		**Intragroup comparison**			**Intergroup comparison**		
	Mean	SD	Mean	SD	Mean	SD	A	B	C	A-B	A-C	B-C

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DAY 1	78.03	3.36	67.79	3.41	64.11	1.68	**	***	***	***	***	NS
DAY 3	80.97	2.32	77.10	2.78	66.90	1.56	**	***	***	***	***	NS
DAY 5	89.95	3.43	77.10	2.78	72.86	1.87	**	***	***	***	***	*
DAY 7	94.07	3.00	77.74	3.22	75.06	8.98	**	***	***	***	***	NS

Multiple comparison ANOVA test confirmed by Bonferronni post-hoc test was used.
*P <0.05; **P =0.001; ***P <0.001; NS, Not significant

Table 1 Cell viability comparison among direct 3D printed aligner and Invisalign trays

Fig.1

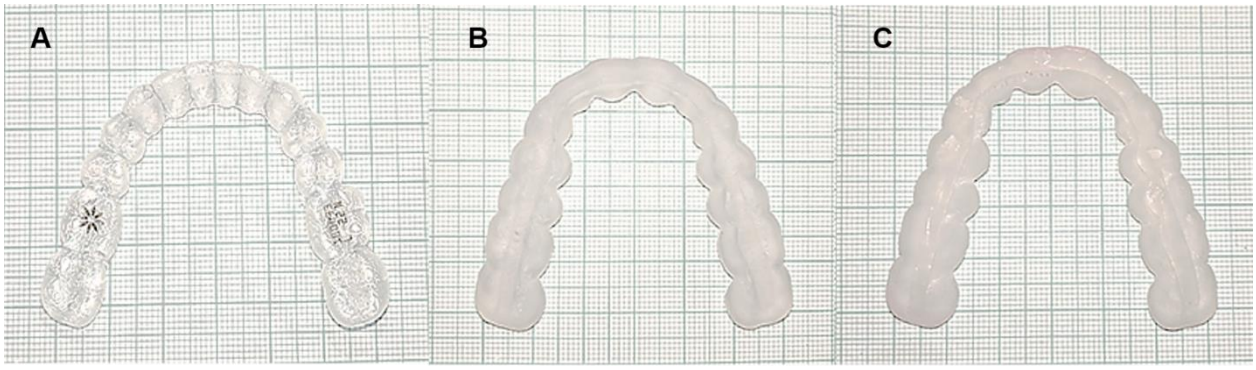

Fig.2

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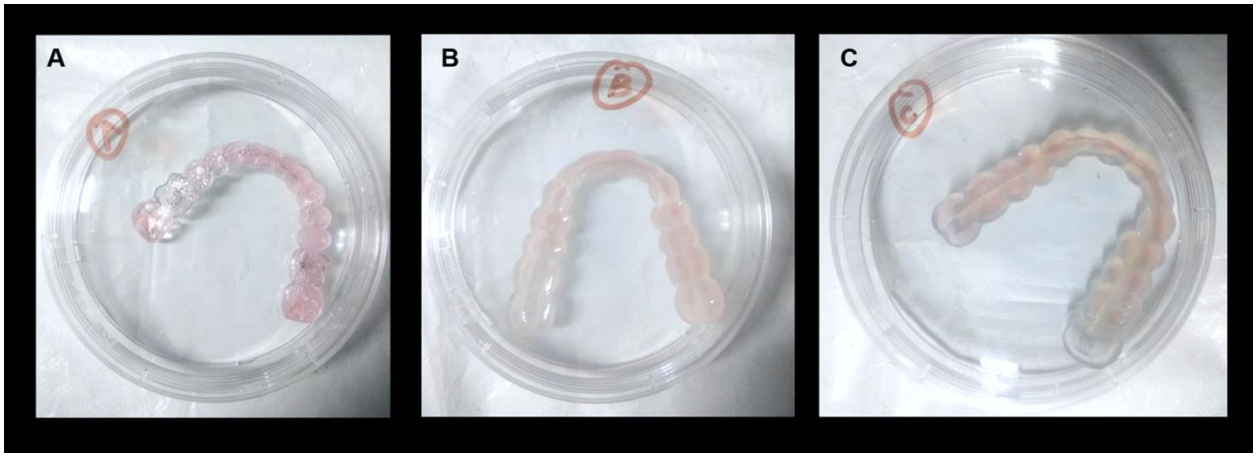

Fig.3

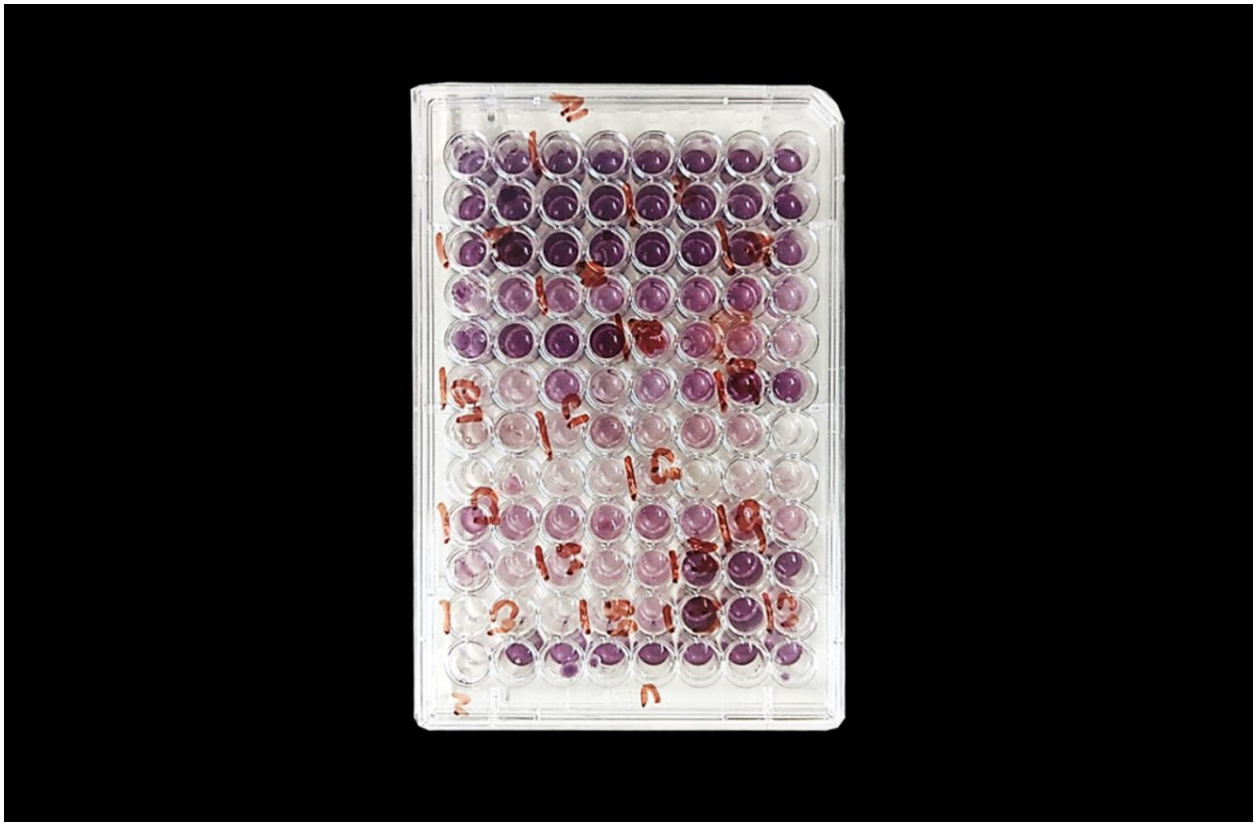

Fig.4

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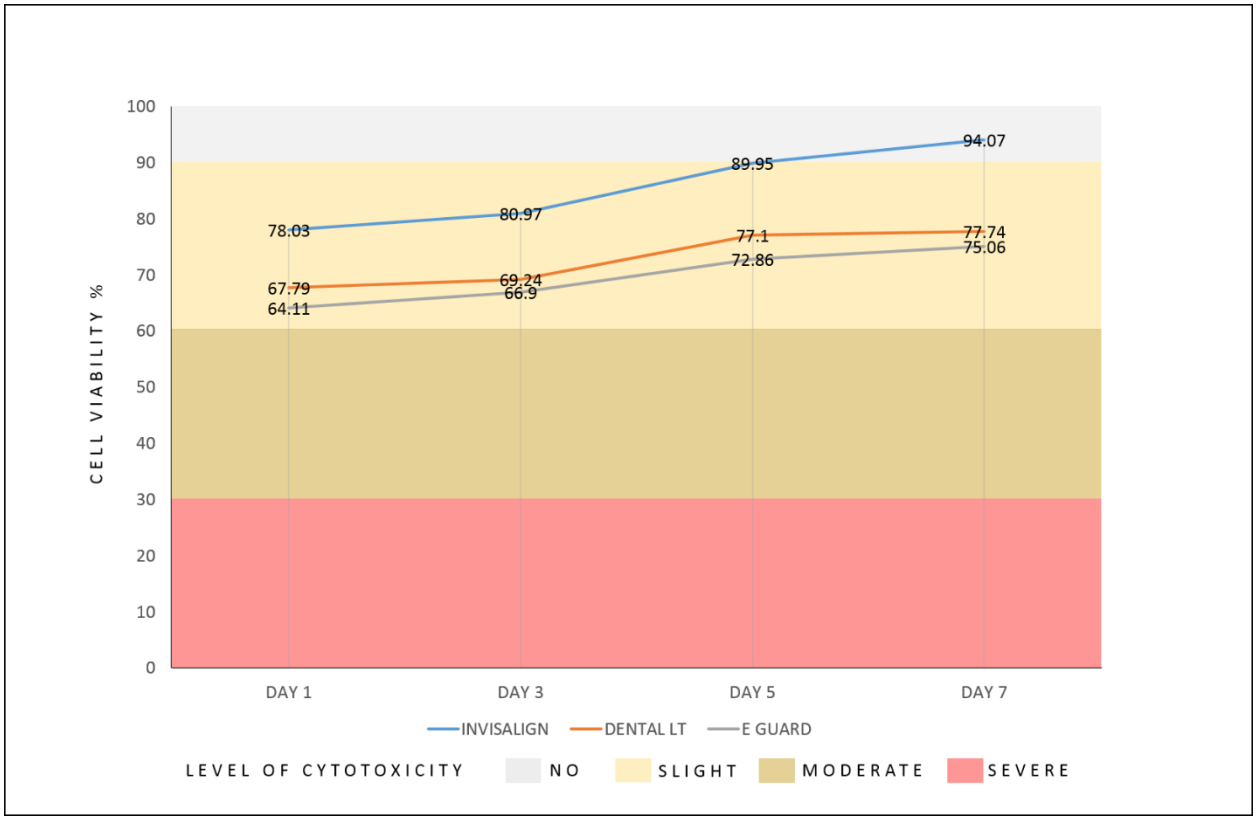

Fig.1 Surface area measurement to quantify the amount of extraction medium needed for each sample (ISO 10993-5 and 10993-12). A. Invisalign tray. B. Dental LT (Long Term) tray. C. E-Guard clear tray.

Fig.2 Dulbecco modified Eagle medium (DMEM)changed at 1st, 3rd, 5th, and 7th day.

Fig.3 Day 8, after 24-hour incubation of 96 wells microplate, MTT assay done using the MTT assay kit.

Fig.4 Level of cytotoxicity at different time intervals [23].