CYTOTOXICITY OF 3D PRINTED MATERIALS AN IN-VITRO STUDY

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfillment for the degree of

MASTER OF DENTAL SURGERY

BRANCH V

DEPARTMENT OF ORTHODONTICS

2016 - 2019

CERTIFICATE

This is to certify that this dissertation titled **"CYTOTOXICITY OF 3D PRINTED MATERIALS - AN IN-VITRO STUDY"** is a bonafide work done by **Dr. S. MOHNISH KUMAR** under my guidance during his post graduate study period between 2016 – 2019.

This dissertation is submitted to **THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY** in partial fulfilment for the degree of **Master of Dental Surgery,** in Branch V – Orthodontics and Dentofacial Orthopaedics. It has not been submitted either partially or fully for the award of any other degree or diploma.

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CONTENTS

INTRODUCTION

Three-dimensional (3D) printing has been widely used in product manufacturing sector for the past three decades. Ever since stereolithography (SLA) was introduced by Chuck Hull in 1984[1] to the present 3D printing technology has evolved from its infancy due to research focusing on improving printing accuracy and printing speeds. 3D printers were initially used in engineering for accurate manufacturing of mechanical parts. In dentistry, 3D printers have been put to use for the last 10 years in the production of clear aligners, dental crown casting, surgical models, splints, dentures and diagnostic models. A scanner or a modelling software is used to create a digital file of the object in standard tessellation language (STL), the global format for 3D printing files. The software then breaks down the object into small layers of 16-300 microns each, known as "build layers".

The time required to produce 3D models depends on the number of layers being printed. 3D manufacturing can be additive (Stereolithography, Fused Deposition Modelling, Selective Laser Sintering, Digital Light Processing, etc.,) or subtractive (e.g. Computer aided designing & computer aided manufacturing /CAD-CAM milling of a ceramic crown). Also known as additive manufacturing, 3D printing is a technology whereby sequential layers of material are deposited on top of one another to eventually form an object. A stereolithography apparatus uses a scanning laser to build parts one layer at a time, in a vat of light-cured photopolymer resin. Each layer is traced-out by the laser on the surface of the liquid resin, at which point a 'build platform' descends, and another layer of resin is wiped over the surface, and the process repeated[2].

3D printing for the past decade has been gaining popularity in orthodontics, ever since this technology has been used in model and appliance fabrication. Today, 3D printed digital splints[3], surgical guides[4], digital functional appliance and maxillary expanders[5]

used in the treatment of malocclusion. 3D printed appliances for intra-oral applications need to be in accordance with biocompatibility standards. For the past 2 decades, Invisalign® (San Jose, California) uses polyurethane[6] for its aligner fabrication combined with SLA printers. SLA printed Dental LT® resin (Form labs Inc.), photo polymeric clear methacrylate-based (methacrylate oligomer and glycol methacrylate) resin made available for appliance fabrication considered to have long-term biocompatibility. Accura 60® SLA (3D systems) a polycarbonate-based SLA material is also available for CAD appliance fabrication. As the 3D printing evolves so are the 3D materials, studies should evaluate their toxicity for safer intra-oral usage.

There are several cytotoxicity assay techniques like Tetrazolium reduction assay, resazurin reduction assay, ATP assay, etc.[7] Tetrazolium reduction assay technology has been widely adopted and remains popular in academic labs as evidenced by thousands of published articles. Various tetrazolium reduction techniques are MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), MTS(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), WST(1)(Water Soluble Tetrazolium). MTT is a positively charged substance and readily penetrates viable eukaryotic cells but MTS, XTT and WST (1) are negatively charged and need an intermediate electron acceptor to penetrate cells.[8] Because of the potential cytotoxicity of intermediate electron acceptors, these must be optimized for various cell lines.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay is a colorimetric assay based on assessing the cell metabolic activity. The MTT reduction assay is one of the commonly used to quantify cell death and cytotoxicity. Viable cells with active metabolism convert MTT into a purple colored formazan

product[8]. When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells.

Although there is an advent of technology, any material should be biocompatible before it is brought to commercial use. There are only a limited amount of studies done previously to assess the cytotoxicity of various orthodontic materials especially polyurethane (Invisalign®)[9-13] and Polycarbonate (brackets and arch wires). Hence it pushed me to investigate the cytotoxicity of these newer plastic materials and to provide for the orthodontic science.

AIMS AND OBJECTIVES

Aim

The aim of this present study is to evaluate the cytotoxicity of stereolithographic 3D printing materials for varying time intervals using MTT assay and application of these materials for intra-oral usage.

Objectives

Objectives of this study were to compare the cytotoxicity of three different stereolithographic 3D printing materials at specific time intervals and to assess the biocompatible of these materials for intraoral usage.

REVIEW OF LITERATURE

In a review article on various aspects of rapid prototyping and manufacturing, by Xue Yan and P GU 1996[14], the basic process of Rapid prototyping and manufacturing has been described. This article provides a picture of various techniques like stereolithography (SLA), Selective laser sintering (SLS), fused deposition modelling (FDM), laminated object manufacturing (LOM), Photo-masking and 3D printing. This article was published in a time at which rapid prototyping was still at its infancy and it discussed various problems faced in RP&M as with any newer technology still in its infancy. The authors suggested further research aimed at improving accuracy, material variety and reducing cost and making it affordable.

D.T. Pham, 1998[15] gave an overview of various rapid prototyping techniques with a schematic diagram for each technique. The authors have mentioned classification of rapid prototyping and manufacturing. Pros and cons of different techniques have been detailed. The authors have also come up with a material selection flowchart for rapid prototyping and manufacturing process

In a review article by J.P. Kruth- 1998[16] a decade of research in Rapid Prototyping has been summarized. The scepticism surrounding the use of rapid prototyping in the early 90s stating that RPM is slow and inaccurate but with constant research and development most of these problems have been overcome. The various processes which are used commercially (SLA, FDM, inkjet printing, 3 D printing, SLS, SLS, Laminated Object Manufacturing) and the ones which are in RND stage (pre-commercial). (selective laser chemical vapour deposition) have been explained with pictorial representation.

BPA (bisphenol A) and hydroquinone (HQ) are present in dental resin materials, and small quantities of these substances may be eluted from the resins. In an in-vitro study done by Terasaka et al, 2005[17] the apoptotic potential of BPA and HQ leached from

dental resins was evaluated to explain the mechanism by which they bring about the cell death. BPA showed a higher induction period (antioxidant activity) but did not cause oxygen uptake. BPA induced internucleosomal DNA fragmentation, a biochemical marker of apoptosis. BPA activated caspase suggesting induction of apoptosis via caspase activation.

Jorge Faber et al, 2006[18] explored the use of 3D printing technology in diagnosis and treatment planning of a patient with an impacted canine. A patient was treated with this technique and the results were shown in the study. CT image of a patient with impacted canine was used for fabrication of a model using rapid prototyping procedure. In addition to bean aid in diagnosis and surgical planning, this model was also helpful in creating a custom attachment for the impacted canine. 3D fabricated attachment was bonded on to the impacted teeth and aided it its eruption. Prototyping could become a new tool for fabricating brackets and other precision accessories for specific needs.

In a report by Mark Lauren et al, 2008[19] computer-based design and production of occlusal splints has been described. Patient 3d model along with bite was used for fabricating the occlusal splint. This 3d model helped in diagnosing articulation problems and in designing a customized splint. Clinically, digital splints reduce the average time needed for placement because intraoral equilibration is minimized.

In a cytotoxicity study done by Theodore Eliades at al, 2009[12] in which he evaluated the cytotoxicity and estrogenic properties of Invisalign® material on human gingival fibroblast using MTT assay. He also assessed the estrogenicity of Invisalign® material on MCF-7 (human adenocarcinoma cell line). Normal saline was used as extraction medium for Invisalign® material and the eluents were diluted to 3 concentrations (5%, 10%, and 20% vol/vol) for assessing cytotoxicity and estrogenicity.

Estrogenicity was assessed by measuring the effect eluents on the proliferation of the estrogen-responsive MCF-7 breast cancer cells. The results of this study concurred that there was no evidence Invisalign® trays having cytotoxic and estrogenic properties within the limits of this study.

In-vitro cytotoxicity of commonly used orthodontic bonding materials were assessed by Ahrari et al, 2010[20]. Samples included a no-mix (Unite), a light-cured (Transbond XT), and a flowable (Denfil Flow) adhesives. Samples were prepared according to ISO standards in the form of discs. Cell culture medium- DMEM served as the extraction medium in which the sample discs were introduced for 1, 3, 5 and 7 days. After each day interval the extraction medium was removed and stored, and new extraction medium was replaced to the sample. MTT assay was carried over to assess cell viability. No mix adhesives showed moderate cytotoxicity on day 1, while light-cured and flow adhesives were not cytotoxic. This study concluded that, care should be taken to protect dentists and patients when no mix adhesives are being handled. Despite higher resin components, the flowable adhesive showed excellent biocompatibility.

Pawlawska et al, 2010[21] conducted a genotoxicity study on common methacrylates used in dentistry. Methacrylate resins are viscous substances that are converted into solid material via polymerization. This process, however, may be incomplete, leading to the release of monomers into the oral cavity and the pulp, which can be reached through the dentin micro-channels. This opens the opportunity for the monomers to reach the bloodstream to cause cellular damage, so it is justified to study their potential toxic effects. In this study the author investigated the cytotoxicity and genotoxicity of 2-hydroxyethyl methacrylate (HEMA) in human peripheral blood lymphocytes and A549 lung-tumor cells. HEMA induced concentration-dependent DNA damage in lymphocytes. The results obtained in this study suggest that HEMA induces

adverse biological effects, mainly via reactive oxygen species, which can lead to DNA damage, apoptosis and cell-cycle delay.

Vitral et al, 2010[22] assessed cellular viability by MTT assay in a murine macrophage cell line J774 with esthetic polycarbonate brackets and quantify nitric oxide production by these macrophages. Cell cultures were evaluated at 3-time intervals: 24, 48, and 72 hours. Cellular viability in all groups was higher at 72 hours compared with 24 hours. Nitric oxide production was significantly greater in all groups at final time. There was significant difference between the final means of the bracket groups and the control group showing the cytotoxic potential of polycarbonate brackets.

Kopperud et al, 2011[23] did a study to analyze leachable monomers and degradation products from polymer-based orthodontic base-plate materials (Heat-cured resin, light-cured and thermoplastic material). Elution was performed in water for 10 days and extract medium was changed and analyzed daily using chromatographic methods (gas chromatography). In this in vitro study, minimal leaching was found from the thermoplastic materials, while leaching of methacrylates was observed from the powder-and-liquid type and the paste material. This study suggests usage of prefabricated thermoplastic plates for patients with an allergy to methacrylates.

In an in-vitro study was conducted by Firat Ozturk et al, 2011[9] to evaluate the cytotoxicity of orthodontic acrylic materials. Gingival samples of systemically health subjects who reported for fibroblasts were isolated from the gingival connective tissue of systemically healthy subjects who reported for crown lengthening procedure. These tissues were cultured to obtain gingival fibroblast cells on which the cytotoxicity study was conducted. Samples were incubated in DMEM for 72 hours. Once the cells were plated in 96 well plate (2000 cells/well) cytotoxicity was assessed using the xCELLigence

system which is an Impedance based real-time cell analyzer. The results infer that the length of the cycle leads to greater cytotoxicity of the tested materials. The study also suggested that there was no significant difference between the spray-on and doughing methods on cytotoxicity.

In a study done by Lingling Qiu et al, 2012[23] CBCT images of patients which were taken for miniscrew placement. They designed a surgical stent using the CBCT data and printed it using stereolithography (SLA). They compared accuracy of freehand placement and placement of TADs using 3D printed stents. 3D CBCT image-based SLA-fabricated surgical stents with enough accuracy for miniscrew implantation could be made available. This method may be more beneficial when patients have insufficient space for freehand insertion: for example, patients with multiple impacted teeth or with limited interradicular distance on account of an extended maxillary sinus

Retamoso et al, 2012[10] conducted a study evaluating the cytotoxicity of esthetic, metallic, and nickel-free orthodontic brackets. Cytotoxicity was assessed on 3T3 mouse fibroblast cell line. Division of study samples are as follows- 11 groups: cellular control, negative control, positive control, metallic, polycarbonate, 2 types of monocrystalline ceramic, 3 types of nickel free, and polycrystalline ceramic brackets. After cell culture mice fibroblasts were plated of 96 wells microplate and the specimens were directly introduced on to these cells. After 24-hour incubation in 5% carbon dioxide at 37°C cytotoxicity was analyzed qualitatively and quantitatively. An inverted light microscope was used to assess cell growth and MTT assay was used to assess cell viability. Different brackets had different ranges of cytotoxicity with Nickel-free brackets exhibiting the better of biocompatibility comparatively. Polycarbonate brackets were highly cytotoxic material for the cells analyzed.

Matthew G. Wiranto et al, 2013[25] assessed the validity, reliability, and reproducibility of digital models obtained from a Chair-side intraoral scanner and cone-beam computed tomography scans of alginate impressions. Bolton analysis was done on both these scanned models. This was then compared to the original poster model. The author suggested that tooth measurement changes between plaster models and scanned models were not statistically significant. They suggested that both CBCT scan of alginate impressions and intraoral scanning are reliable methods as a physical plaster model and could be used in diagnosis and treatment planning.

Kloukos et al 2013[26], evaluated the biological effects of water eluents from polycarbonate based esthetic orthodontic brackets. The brackets' composition was analyzed by spectrometry. The cytotoxicity and estrogenicity of the eluents obtained after 3 months storage of the brackets in water were investigated in murine fibroblasts (NIH 3T3), breast (MCF-7) and cervical cancer (CCl-2/Hela) cell lines. The study reported significant induction of cell death and a concurrent decrease in cell proliferation. Moreover, increased eluent significantly reduced the levels of the estrogen signalling associated gene pS2, specifically in MCF7 cells, suggesting that cell death induced by this material is associated with downregulation of estrogen signalling pathways.

The metal alloys commonly used in dental practice have been debated over the effect they have in the oral cavity. Rusu et al, 2014[11] assessed the cytotoxicity of Ni-Cr and Co-Cr alloy on human dermal fibroblast. The cultured both commercially available immortalized cell line and dermal fibroblasts obtained from human skin tissues (primary culture). Eluates from both samples and the sample itself were introduced to these cultured cells. The cells were observed daily using an inverted light microscope. Commercial cell lines had a better cell density of fusiform fibroblasts than primary

culture. This study concluded that both Ni-Cr and Co-Cr did not have any significant cytotoxicity and that it could be used in day-to-day dental practice.

Thyagaseely Premaraj et al, 2014[6] did an in-vitro cytotoxic study on Invisalign® plastic aligners. They assessed the cellular behavior of oral epithelial cells when exposed to Invisalign® material. cellular responses of oral epithelium exposed to Invisalign® plastic in vitro was evaluated. Invisalign® material was soaked in artificial saliva and saline for 2, 4 and 8 weeks. Human keratinocyte cells were exposed to eluates. Cells grown in media containing saline solution or saliva served as controls. MTT assay and flow cytometry were done to assess the viability of cells and membrane integrity, respectively. Cell-substrate impedance sensing was done to assess cellular adhesion and micromotion of epithelial cells. Cells exposed to saline-solution eluate showed signs of decreased cell viability, increased membrane permeability and decreased cell adhesion whereas saliva eluates did not induce significant changes when compared to control. Exposure to Invisalign® plastic caused changes in viability, membrane permeability, and adhesion of epithelial cells in a saline-solution environment. The results of this study suggest that isocyanate from polyurethane material might cause allergic reactions in case of microleakage and hapten formation secondary to compromised epithelial integrity. However, these results also suggest that saliva might offer protection.

In a review article by Groth et al 2014[1] various 3 D printing techniques such as stereolithography (SLA), fused deposition modelling (FDM), digital light processing (DLP) and polyjet photo-polymerization (PPP) has been explained. They have also explained about 3 D printers introduced in orthodontics and has listed a few materials used in orthodontics most of which are ABS-like plastic resins, acrylics, polylactic acid (PLA)

Laurence W. McKeen, 2014[27] elaborated medical devices range from simple devices to test equipment and to implants. Plastics are used more and more in these devices, for weight, cost, and performance purposes. Examples of medical devices include surgical instruments, catheters, coronary stents, pacemakers, magnetic resonance imaging (MRI) machines, X-ray machines, prosthetic limbs, artificial hips/knees, surgical gloves, and bandages. The first section reviews the general composition of plastic materials which will include the materials added to the basic polymers. The second section discusses many factors that contribute to the plastic selection. The final section reviews the chemistry, the response to sterilization processes, and the application of most common plastic materials in medical products.

Fabricated a resin appliance with incorporated wire component (labial bow and 2 Adam's clasps) without an analogue impression using intraoral scanner and CAD was demonstrated by Noor Al Mortadi et al, 2015[28]. The results showed that the applied techniques may provide new manufacturing and design opportunities in orthodontics and highlights the need for intraoral-specific additive manufacture materials to be produced and tested for biocompatibility compliance. In a trial, the retainer was fitted orally and judged acceptable by the clinician according to the typical criteria when placing such appliances in-situ.

3D printing is gaining popularity by providing a tool for fast, cost-effective, and highly customizable fabrication. However, little is known about the toxicity of 3D-printed objects. In a work by Shirin Mesbah Oskui et al, 2015[29], the toxicity of printed parts from two main classes of commercial 3D printers, fused deposition modelling and stereolithography. The toxicity of these 3D-printed parts using zebra fish (Danio rerio), a

widely used model organism in aquatic toxicology. Zebra fish embryos were exposed to 3D-printed parts and monitored for rates of survival, hatching, and developmental abnormalities. They found that parts from both types of printers were measurably toxic to zebra fish embryos, with STL-printed parts significantly more toxic than FDM-printed parts. They also developed a simple post-printing treatment (exposure to ultraviolet light) that largely mitigates the toxicity of the STL-printed parts.

Bisphenol A (BPA) is an endocrine-disrupting chemical used in the manufacture of many products used daily. In a study done by Elmetwally 2018[30], the effects of BPA on migration and on the expression of some apoptotic genes were examined. The results revealed that BPA decreased migration of oTr1 cells. Regarding apoptosis, expression of the anti-apoptotic gene Bcl-2 mRNA was down-regulated; however, expression of pro-apoptotic genes (Bax, cathepsin B, caspase-3 and c-myc) was reduced at the higher concentrations of BPA. Results of this study suggest that BPA may impair implantation by decreasing migration of oTr1 cells and inhibiting apoptosis.

MATERIALS AND METHODS

This is an in-vitro prospective cytotoxicity study conducted by the Department of Orthodontics and Dentofacial Orthopedics, Sri Ramakrishna Dental College and Hospital, Coimbatore, India.

3D Printed samples:

Three types of stereolithographic 3D printing materials were used for this study namely Accura 60® SLA (3D systems, Rockhill, South Carolina), Dental LT® clear (Form Labs, Somerville, Massachusetts) and Invisalign® (Aligntech, San Jose, California). 3D printed clear aligner tray using Accura 60® SLA (Fig 1) was 3D printed stereolithographically in SLA Viper Si2 System 3D printer (3D systems, Rockhill, South Carolina) (Fig 2). Accura 60® SLA is a polycarbonate-based photo-polymeric resin and was 3D printed at TIFAC core facility located in PSG Institute of Technology, Peelamedu, Coimbatore, India.

Dental LT® (Fig 1) is a methacrylate-based photo polymeric resin. Its printing process involves using Form2 SLA printer (Fig 3) to print the physical tray followed by rinsing the printed part with 96% isopropyl alcohol for 5 minutes to dissolve any uncured resin and finally post-curing with 405nm form cure unit for 20 minutes at 80°C. All this process was done at Form labs, Somerville, Massachusetts, USA.

Third material of choice used in this study was Invisalign® (San Jose, California) (Fig 1). Invisalign® tray of a lower arch for refinement in a patient model was used for the study. Invisalign® tray is made of a 3D printed polyurethane based material. Three materials used in this study were all 3D printed using stereolithography. Stereolithography (SLA) (Fig 4) is an additive manufacturing process which employs a vat of liquid ultraviolet curable photopolymer "resin" and an ultraviolet laser to build parts' layers one at a time. For each layer, the laser beam traces a cross-section of the part pattern on the surface of the liquid resin. Exposure to the ultraviolet laser light cures and

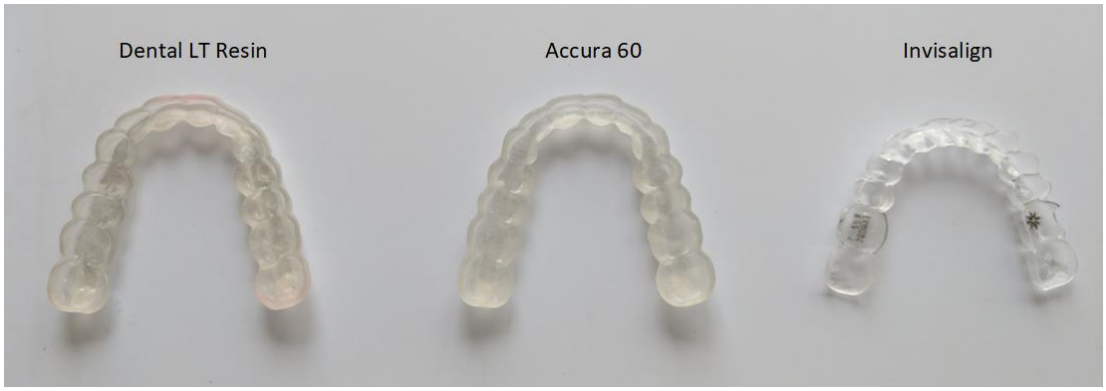


FIGURE 1: 3D Printed samples

FIGURE 2: SLA 3D Printer (SLA Viper 2, 3D systems)

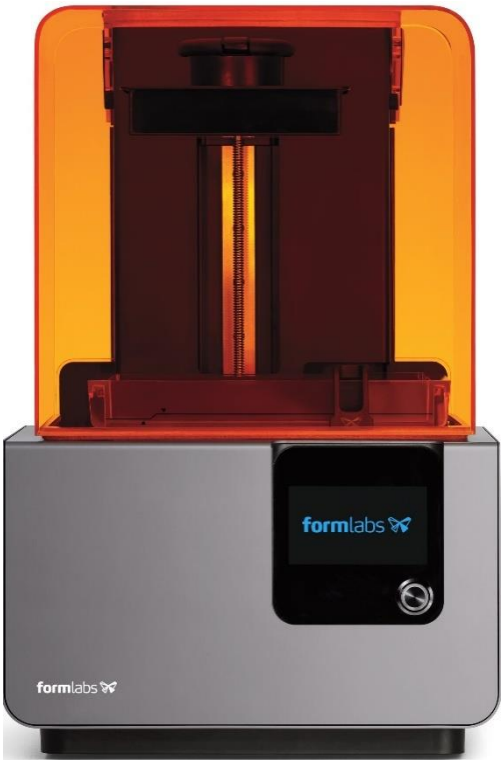


FIGURE 3: Form 2 SLA 3D printer

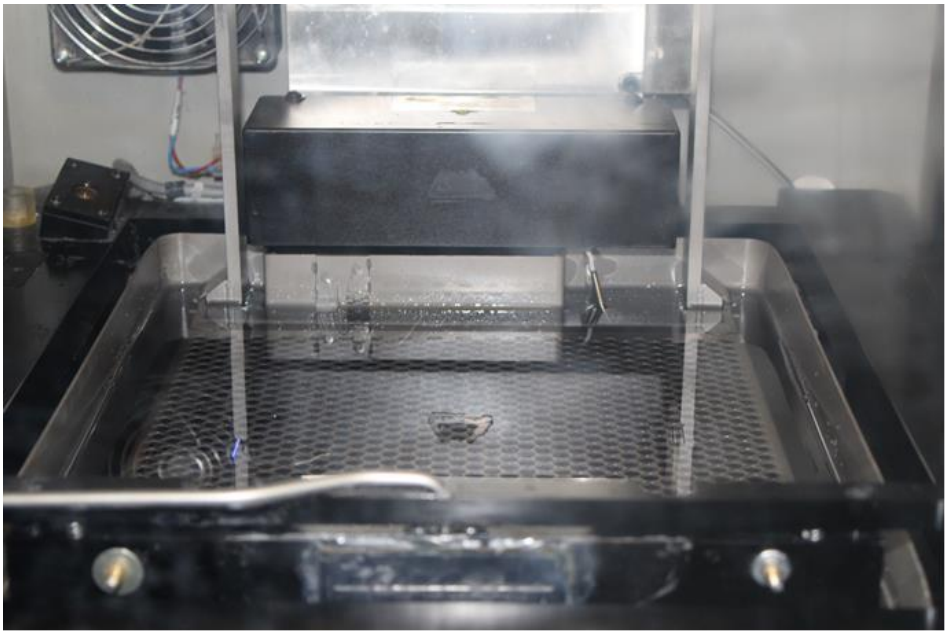


FIGURE 4: SLA 3D printing procedure

solidifies the pattern traced on the resin and joins it to the layer below. This process repeats until the final shape of the sample part is achieved.

Cytotoxicity study:

The cytotoxicity part of the study was conducted in Department of Biotechnology, PSG Institute of Technology, Peelamedu, Coimbatore, India under the guidance of Dr Vidyalakshmi. S, M.Sc., Ph.D., Assistant Professor. It involved 3 processes- Sample preparation, Cell culture and Cytotoxicity assay.

Sample preparation

The surface area covered by splints made of Accura 60® SLA, Dental LT® resin and Invisalign® were measured using a graph paper (Fig 5) according to the international organization for standardization for assessing the cytotoxicity of a medical device (ISO 10993). This was done in order to quantify the amount of extraction medium needed for each of these samples. Splints made of Accura 60® SLA and Dental LT® resin covered 11.96cm^2 and required 2ml extraction medium. Invisalign® covered 8.81 cm^2 and require 1.5 ml of extraction medium. After measuring the surface area covered all 3 sample materials were put in separate sterilization pouches (Capri self-sealing sterilization pouches) and sealed before exposing the samples to UV light for 45 minutes in a UV cabinet (Ideal medical systems, Bangalore, India) (Fig 6, 7). This was done to prevent any bacterial contamination. UV exposed 3D printed samples were then kept in a 100mm petri dish (Corning®) (Fig 8). Culture medium (Dulbecco modified Eagle medium, DMEM (Gibco®, Invitrogen)) served as the extraction medium for this study. 2ml of extraction medium was used for Accura 60® SLA and Dental LT® resin and 1.5ml was used for Invisalign® sample as previously mentioned.

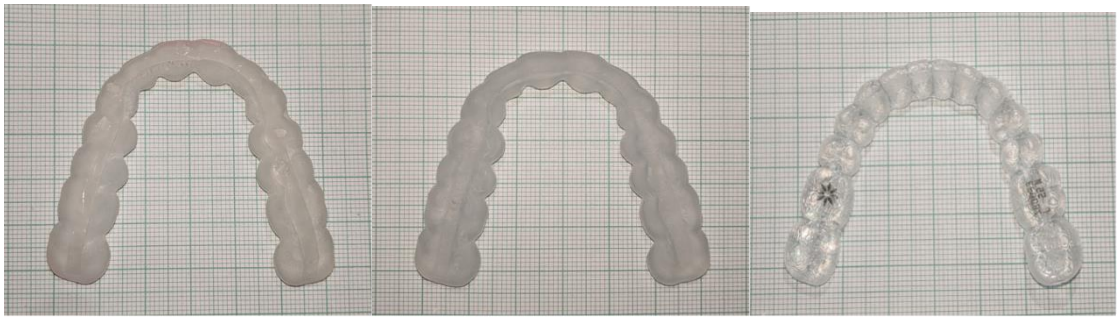


FIGURE 5: Measuring surface area of each sample

FIGURE 6: 3d printed samples in autoclave pouches introduced into UV chambe

FIGURE 7: UV light exposure for 45 mins

FIGURE 8: 100 mm petri dish

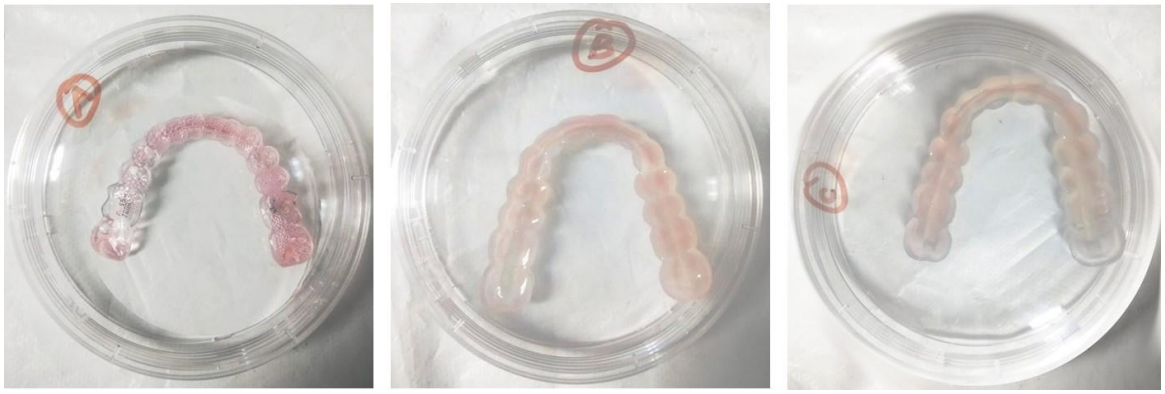


FIGURE 9: Day 1 of culture introduction to sample (sample A- Polyurethane, sample B- Methacrylate, sample C- Polycarbonate)

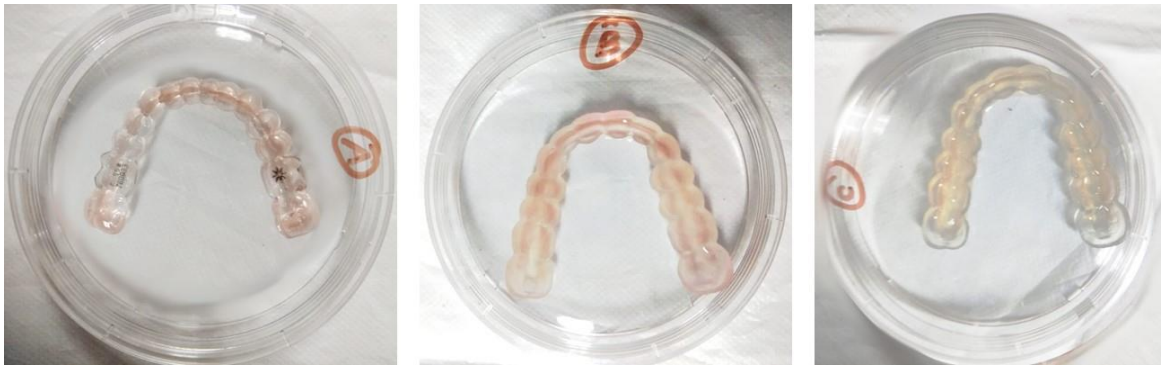


FIGURE 10: Day 3 of culture introduction to sample (sample A- Polyurethane, sample B- Methacrylate, sample C- Polycarbonate)

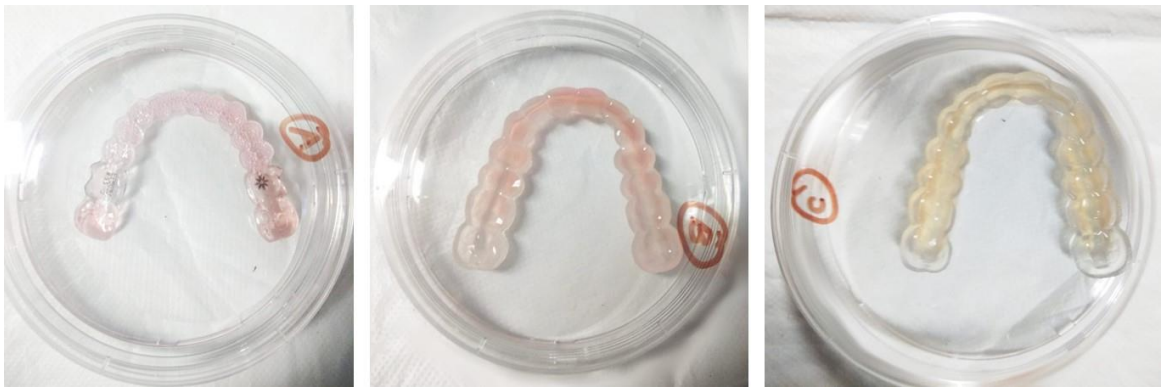


FIGURE 11: Day 5 of of culture introduction to sample (sample A- Polyurethane, sample B- Methacrylate, sample C- Polycarbonate)

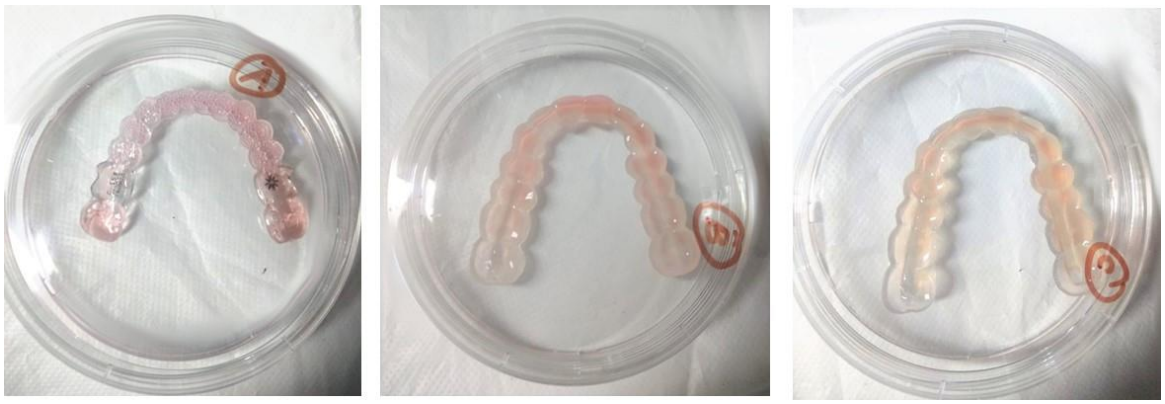


FIGURE 12: Day 7 of culture introduction to sample (sample A- Polyurethane, sample B- Methacrylate, sample C- Polycarbonate)

Extraction medium was changed at 1, 3, 5 and 7 days. After each time interval the culture medium was changed at 1, 3, 5 and 7 days. After each time interval, the culture medium was removed from the 3D printed samples and new culture medium was introduced into the samples. The removed culture medium was then labelled for each time interval (i.e. 1st, 3rd, 5th and 7th day) (Fig 9, 10, 11, 12) and stored at -20 degree Celsius until the commencement of the cytotoxicity study using MTT assay on the 8th day.

Division of samples:

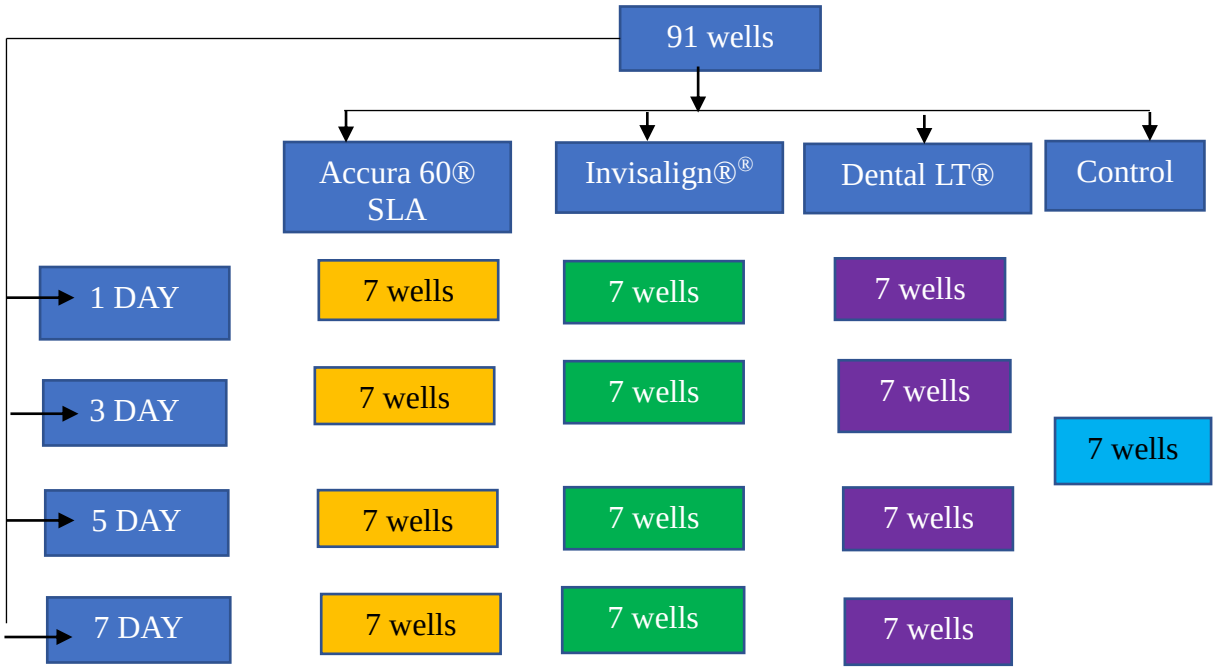


FIGURE 13: Sample division

Cell line culture

Mouse embryonic fibroblast cell lines 3T3 mice fibroblasts were obtained from National centre for cell science, Pune, India. Mouse fibroblast cell line was cultured in Dulbecco's Modified Eagle medium (DMEM) (Himedia) + 5% fetal calf serum (Himedia) + penicillin and streptomycin. The cell line and culture medium are incubated (Fig 14) at 37°C in an atmosphere of 95% air and 5% CO_2 Once the cells attain at 37°C in

FIGURE 14: Incubator

FIGURE 15: 96 well TC grade microplate

at an atmosphere 80% confluence these cells were transferred on to 91 wells of 96 well tissue culture grade plate (Corning®)(Fig 15) Nearly 5,000 cells were seeded per plate in 91 wells TC grade plate along with normal cell culture medium (DMEM).

Cytotoxicity assay:

Mice fibroblasts are 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 is divided for each sample i.e. 28 wells (7 wells each for 1^{st}, 3^{rd}, 5^{th} and 7^{th} day) polycarbonate, 28 wells polyurethane and 28 wells methacrylate and 7 wells serve as control were in cells grow in normal culture medium (Ref Fig. 13). After this, the 96 well plate is incubated for a 24hour period.

After 24-hour incubation, MTT assay is done using MTT assay kit (Merck®). 5µl per well of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium solution 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 91 wells (Fig 16). Optical density was measured in an ELISA plate reader (Biotek technologies) (Fig 17) at 540 nm. Cell viability of these mice fibroblasts were assessed as cell viability percentage using the following formula and the results were tabulated.

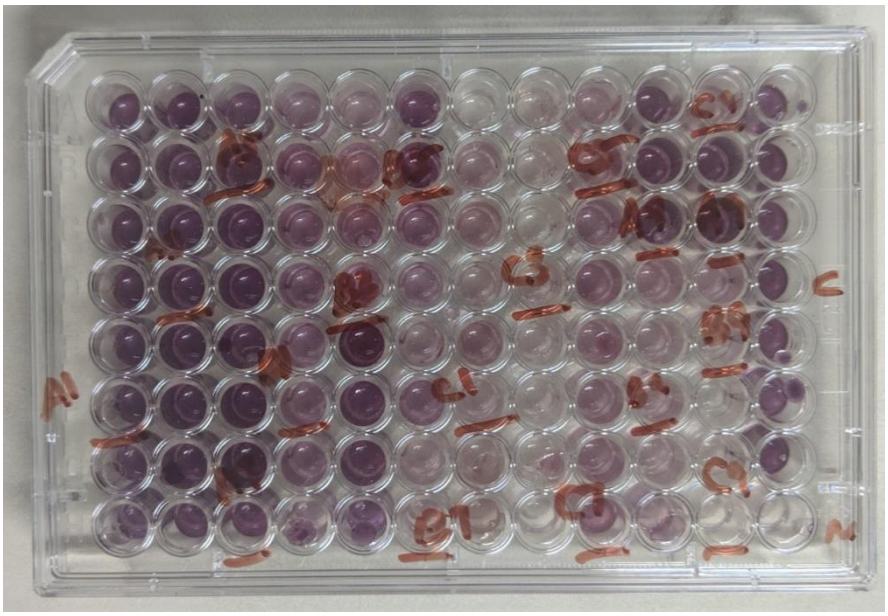


FIGURE 16: 96 well plate after MTT assay

FIGURE 17: ELISA plate reader – Biotek technologies

RESULTS

The aim of this study was to assess the cytotoxicity of three different 3D printed materials for four different time intervals using MTT assay.

91 specimens were divided into 13 groups based on materials and number of days. Control group had 7 samples. Three materials M1, M2 and M3 (Invisalign®, Dental LT® and Accura 60®) had 28 samples each. These three material groups were further subdivided into four groups containing 7 samples each, based on respective time intervals (Day 1, Day 3, Day 5 and Day 7).

In each group mean and standard deviation of cell viability % calculated. The descriptive statistics including mean, standard deviation, standard error and 95% confidence interval for the three materials for 4-time intervals were calculated and tabulated. One-way ANOVA and Tukey test were used for statistical analysis.

Intragroup findings

One-way ANOVA was done to analyze variability in cell viability % of a material for different days (Day 1, 3, 5 and 7). Tukey test was done to compare inter-day differences in cell viability.

Invisalign®

The mean, standard deviation, standard error and 95% confidence interval for Invisalign® is given in table 1. There was statistically insignificant (P-value>0.05) difference for Invisalign® with respect to day variation.

TABLE – 1

ANOVA – Invisalign®

	Sum of Squares	Df	Mean Square	F	P value.
Between Groups	11.311	3	3.770	.125	.945
Within Groups	726.776	24	30.282		
Total	738.088	27			

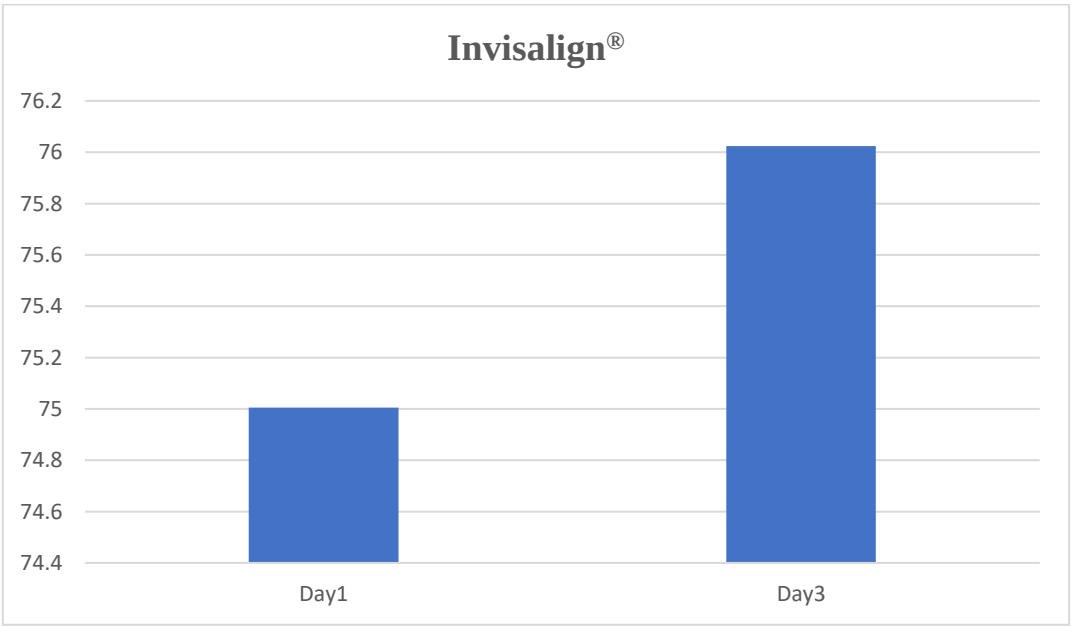
P value < 0.05- S, P value > 0.05- NS

TABLE - 2

Day 1 vs Day 3											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	Sig.
					Lower Bound	Upper Bound					
Day 1	7	74.486	3.8786	1.46596	70.8987	78.0729	70.3	80.29	0.5188	2.9415	0.998
Day 3	7	75.005	3.8771	1.46542	71.4189	78.5904	68.74	78.73			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 1



Comparison of Day 1 vs Day 3 (Invisalign®)

Comparison of cell viability of Invisalign® between Day 1 and Day 3 is given in Graph 1. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 1 and Day 3 analyzed with Tukey test were tabulated in Table 2. There is **no statistically** significant (P value>0.05) difference between Day 1 and Day 3 cell viability of Invisalign®. The results indicate cell viability of day 1 sample to be less when compared to cell viability of day 3 sample. Polyurethane material seems to be more toxic on day 1 than day 3 but the difference is insignificant statistically.

Comparison of Day 1 vs Day 5 (Invisalign®)

Comparison of cell viability of Invisalign® between Day 1 and Day 5 is given in Graph 2. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 1 and Day 5 analyzed with Tukey test is tabulated in Table 3. There is **no statistically significant** (P value>0.05) difference in Day 1 and Day 5 cell viability % of Invisalign®. The results indicate cell viability of day 1 sample to be less when compared to cell viability of day 5 sample. Polyurethane material seems to be more toxic on day 1 than day 5 but the difference is insignificant statistically.

Comparison of Day 1 vs Day 7 (Invisalign®)

Mean cell viability % of Invisalign® between Day 1 and Day 7 is given in Graph 3. The mean, standard deviation, 95% confidence interval, mean difference and P value for Day 1 and Day 7 were analyzed with Tukey test is tabulated in Table 4. There is **no statistically significant** (P value>0.05) difference in Day 1 and Day 7 cell viability values

of Invisalign®. The results indicate cell viability of day 1 sample to be less when compared to cell viability of day 7 sample. Polyurethane material seems to be more toxic on day 1 than day 7 but the difference is insignificant statistically.

TABLE - 3

Day 1 vs Day 5											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	Sig.
					Lower Bound	Upper Bound					
Day 1	7	74.486	3.8786	1.466	70.8987	78.0729	70.3	80.29	1.4082	2.94145	0.963
Day 5	7	75.894	6.9643	2.632	69.4531	82.3349	67.32	86.12			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 2

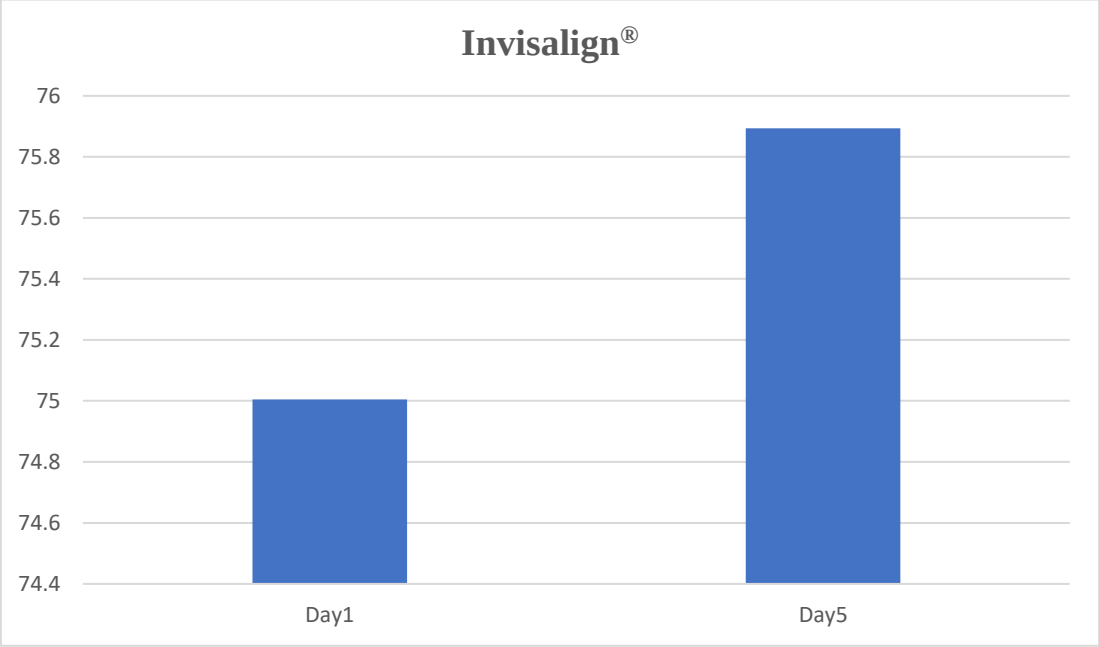


TABLE - 4

Day 1 vs Day 7											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	Sig.
					Lower Bound	Upper Bound					
Day 1	7	74.486	3.8786	1.466	70.8987	78.0729	70.3	80.29	1.53789	2.9415	0.953
Day 7	7	76.024	6.5232	2.4655	69.9908	82.0567	69.91	85.21			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 3

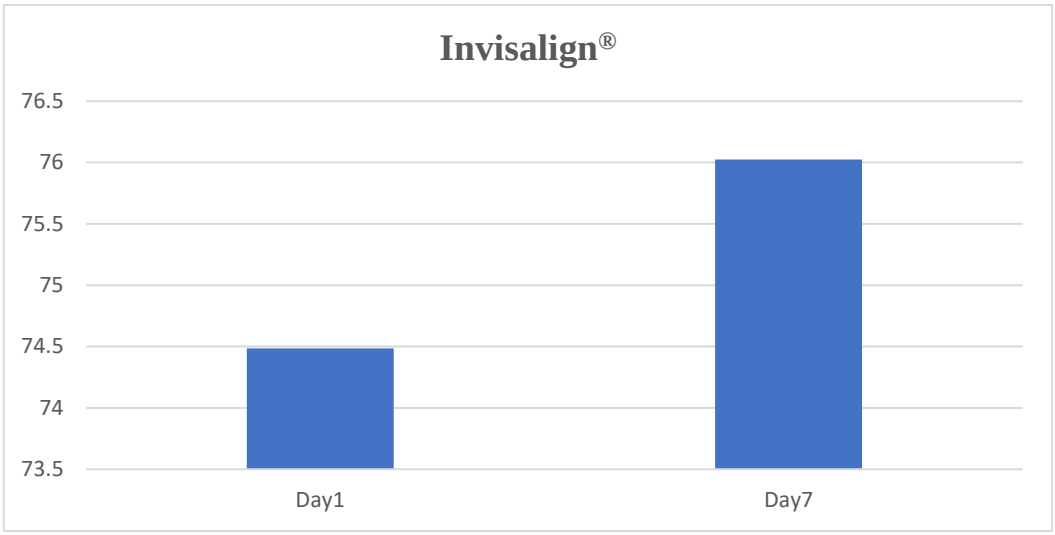
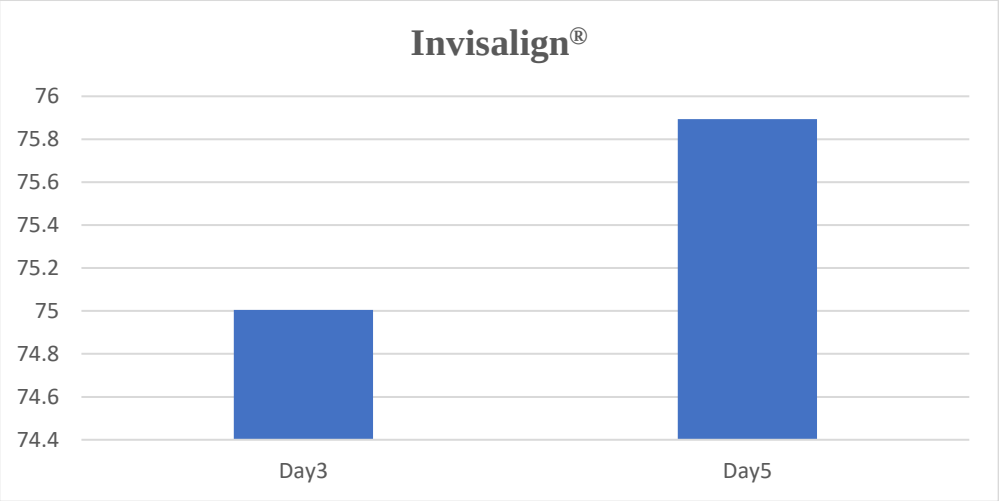


TABLE - 5

Day 3 vs Day 5											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	Sig.
					Lower Bound	Upper Bound					
Day 3	7	75.005	3.8771	1.4654	71.4189	78.5904	68.74	78.73	-0.8894	2.94145	0.99
Day 5	7	75.894	6.9643	2.6323	69.4531	82.3349	67.32	86.12			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 4



Comparison of Day 3 vs Day 5 (Invisalign®)

Mean plots for cell viability of Invisalign® between Day 3 and Day 5 is given in Graph 4. The mean, standard deviation, 95% confidence interval, mean difference and P value for Day 3 and Day 5 analyzed with Tukey test is tabulated in Table 5. There is **no statistically significant** (P-value > 0.05) difference in Day 3 and Day 5 cell viability values of Invisalign®. The results indicate cell viability of day 3 sample to be less when compared to cell viability of day 5 sample. Polyurethane material seems to be more toxic on day 3 than day 5 but the difference is insignificant statistically.

Comparison of Day 3 vs Day 7 (Invisalign®)

Comparison of cell viability of Invisalign® between Day 3 and Day 7 is given in Graph 5. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 3 and Day 7 analyzed with Tukey test is tabulated in Table 6. There is **no statistically significant** (P-value > 0.05) difference in Day 3 and Day 7 cell viability of Invisalign®. The results indicate cell viability of day 3 sample to be less when compared to cell viability of day 7 sample. Polyurethane material seems to be more toxic on day 3 than day 7 but the difference is insignificant statistically.

Comparison of Day 5 vs Day 7 (Invisalign®)

Comparison of cell viability of Invisalign® between Day 5 and Day 7 is given in Graph 6. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 5 and Day 7 analyzed with Tukey test is tabulated in Table 7. There is **no statistically significant** (P-value 0.05) difference in Day 5 and Day 7 cell viability of Invisalign®. The results indicate cell viability of day 5 sample to be less

when compared to cell viability of day 7 sample. Polyurethane material seems to be more toxic on day 5 than day 7 but the difference is insignificant statistically.

TABLE - 6

Day 3 vs Day 7											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	Sig.
					Lower Bound	Upper Bound					
Day 3	7	75.005	3.8771	1.4654	71.4189	78.5904	68.74	78.73	-1.0191	2.9415	0.985
Day 7	7	76.024	6.5232	2.4655	69.9908	82.0567	69.91	85.21			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 5

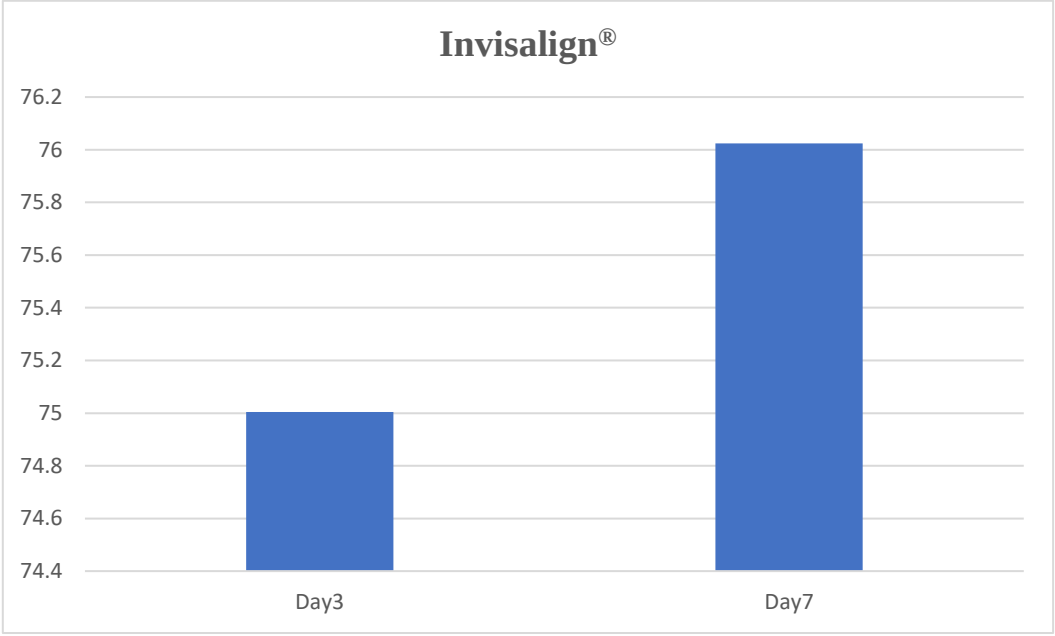
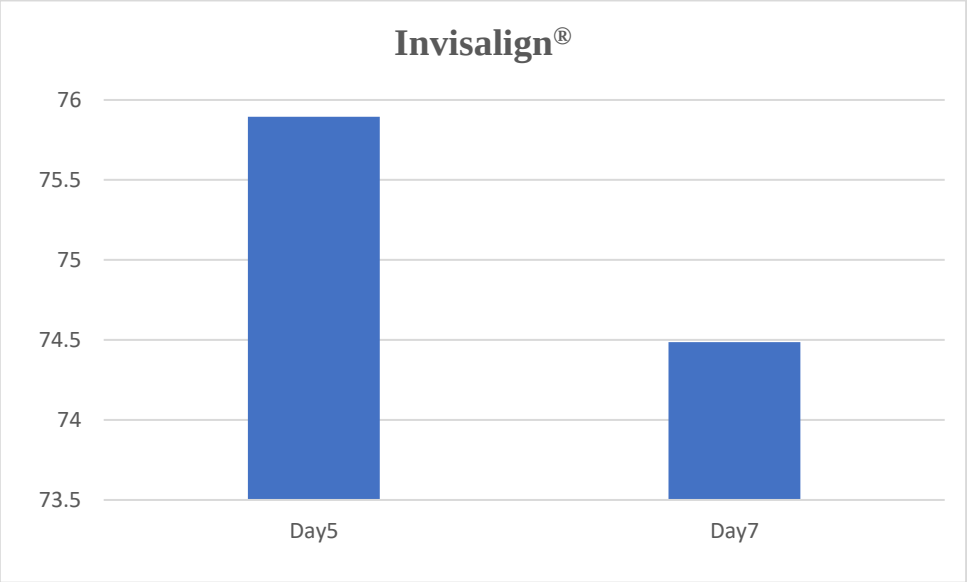


TABLE - 7

Day 5 vs Day 7											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	Sig.
					Lower Bound	Upper Bound					
Day 5	7	75.894	6.96432	2.63227	69.4531	82.3349	67.32	86.12	-0.1297	2.94145	1.000
Day 7	7	76.0237	6.5232	2.46554	69.9908	82.0567	69.91	85.21			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 6



For Invisalign®, the material cytotoxicity was comparatively more on day 1 and as days progressed, there was a reduction in cytotoxicity as shown in the above-mentioned tables and graphs, but the differences are not statistically significant.

Dental LT®

Comparison of Day 1 vs Day 3

Comparison of cell viability of Dental LT® between Day 1 and Day 3 is given in Graph 7. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 1 and Day 3 analyzed with Tukey test is tabulated in Table 8. There is **no statistically significant** (P value>0.05) difference in Day 1 and Day 3 cell viability of Dental LT®. The results indicate cell viability of day 1 sample to be less when compared to cell viability of day 3 sample. Methacrylate material seems to be more toxic on day 1 than day 3 but the difference is insignificant statistically.

Comparison of Day 1 vs Day 5 (Dental LT®)

Comparison of cell viability of Dental LT® between Day 1 and Day 5 is given in Graph 8. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 1 and Day 5 analyzed with Tukey test is tabulated in Table 9. There is **no statistically significant** (P value>0.05) difference in Day 1 and Day 5 cell viability of Dental LT®. The results indicate cell viability of day 1 sample to be less when compared to cell viability of day 5 sample. Methacrylate material seems to be more toxic on day 1 than day 5 but the difference is insignificant statistically.

Comparison of Day 1 vs Day 7 (Dental LT®)

Comparison of cell viability of Dental LT® between Day 1 and Day 7 is given in Graph 9. The mean, standard deviation, standard error and 95% confidence interval,

mean difference and P value for Day 1 and Day 7 analyzed with Tukey test is tabulated in Table

TABLE - 8

Day 1 vs Day 3											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	P value
					Lower Bound	Upper Bound					
Day 1	7	60.256	3.8087	1.4395	56.7333	63.7781	54.6	64.2	-0.46322	1.6453	0.992
Day 3	7	60.719	2.6352	0.996	58.2817	63.1561	56.29	64.2			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 7

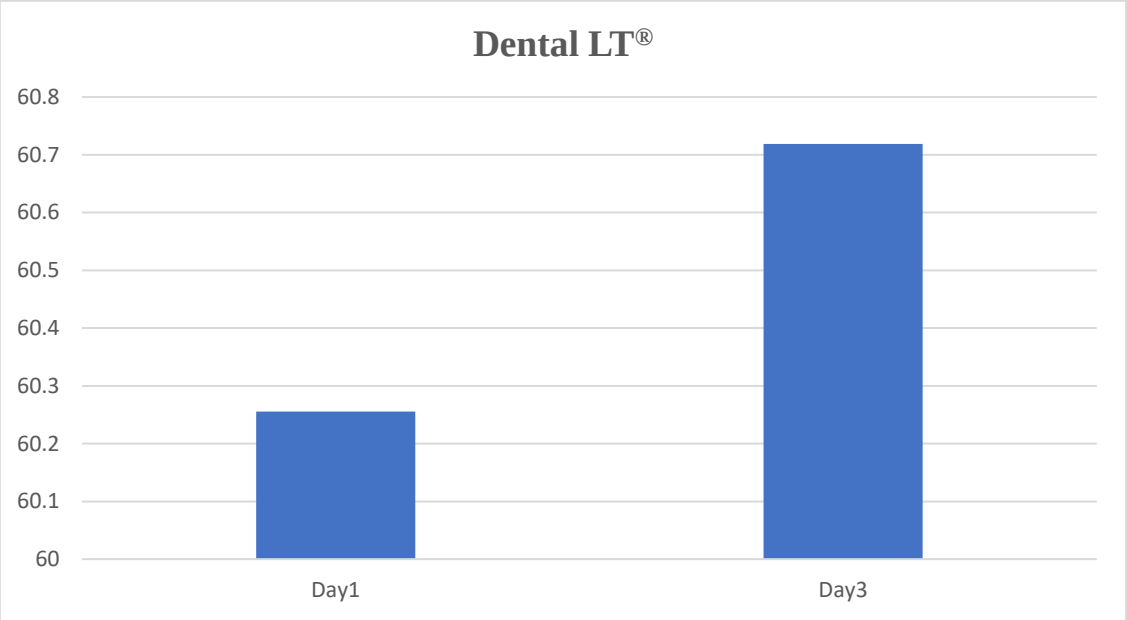


TABLE - 9

Day 1 vs Day 5											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	P value
					Lower Bound	Upper Bound					
Day 1	7	60.256	3.8087	1.4395	56.7333	63.7781	54.6	64.2	-0.53734	1.6453	0.988
Day 5	7	60.793	3.1502	1.1907	57.8796	63.7065	56.94	64.72			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 8

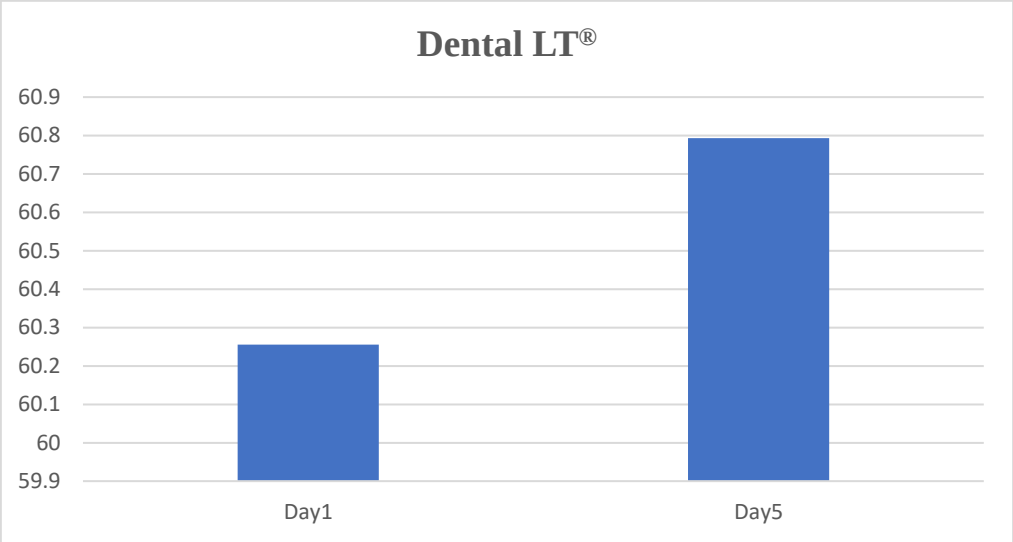
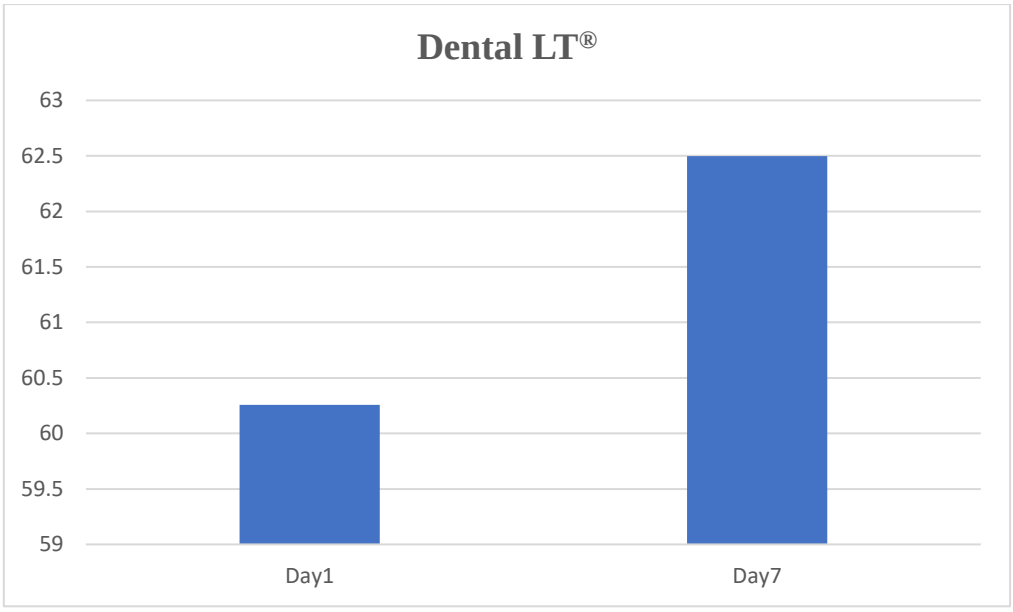


TABLE -10

Day 1 vs Day 7											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	P value
					Lower Bound	Upper Bound					
Day 1	7	60.256	3.8087	1.4395	56.7333	63.7781	54.6	64.2	-2.24199	1.6453	0.534
Day 7	7	62.498	2.5542	0.9654	60.1354	64.8599	59.01	66.67			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 9


Dental LT®
63
62.5
62
61.5
61
60.5
60
59.5
59
Day1
Day7

10. There is **no statistically significant** (P value>0.05) difference in Day 1 and Day 7 cell viability of Dental LT®. The results indicate cell viability of day 1 sample to be less when compared to cell viability of day 7 sample. Methacrylate material seems to be more toxic on day 1 than day 7 but the difference is insignificant statistically.

Comparison of Day 3 vs Day 5 (Dental LT®)

Comparison of cell viability of Dental LT® between Day 3 and Day 5 is given in Graph 10. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 3 and Day 5 analyzed with Tukey test is tabulated in Table 11. There is **no statistically significant** (P-value 0.05) difference in Day 3 and Day 5 cell viability of Dental LT®. The results indicate cell viability of day 3 sample to be less when compared to cell viability of day 5 sample. Methacrylate material seems to be more toxic on day 3 than day 5 but the difference is insignificant statistically.

Comparison of Day 3 vs Day 7 (Dental LT®)

Comparison of cell viability of Dental LT® between Day 3 and Day 7 is given in Graph 11. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 3 and Day 7 analyzed with Tukey test is tabulated in Table 12. There is **no statistically significant** (P-value 0.05) difference in Day 3 and Day 7 cell viability of Dental LT®. The results indicate cell viability of day 3 sample to be less when compared to cell viability of day 7 sample. Methacrylate material seems to be more toxic on day 3 than day 7 but the difference is insignificant statistically.

TABLE - 11

Day 3 vs Day 5											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	P value
					Lower Bound	Upper Bound					
Day 3	7	60.719	2.6352	0.996	58.2817	63.1561	56.29	64.2	-0.07412	1.6453	1
Day 5	7	60.793	3.1502	1.1907	57.8796	63.7065	56.94	64.72			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 10

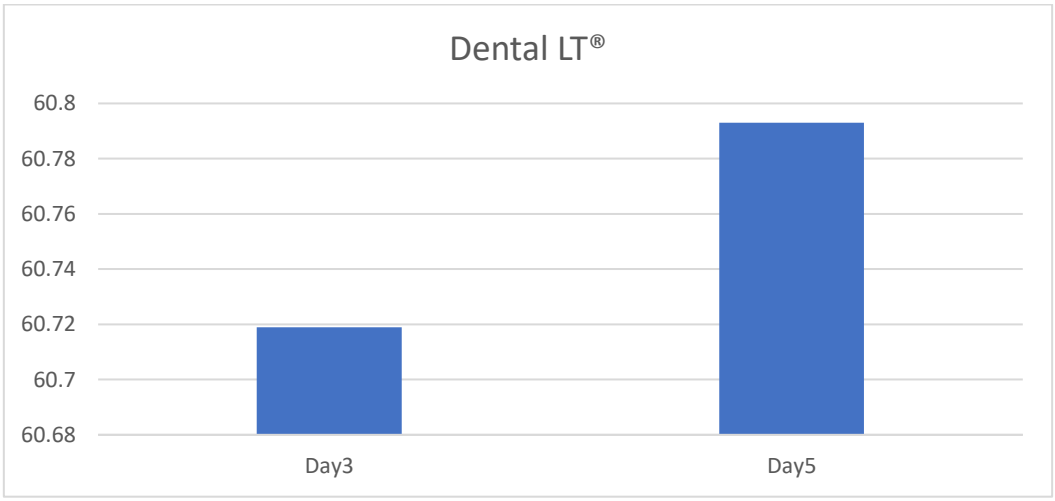


TABLE - 12

Day 3 vs Day 7											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	P value
					Lower Bound	Upper Bound					
Day 3	7	60.719	2.6352	0.996	58.2817	63.1561	56.29	64.2	-1.77877	1.6453	0.704
Day 7	7	62.498	2.5542	0.9654	60.1354	64.8599	59.01	66.67			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 11

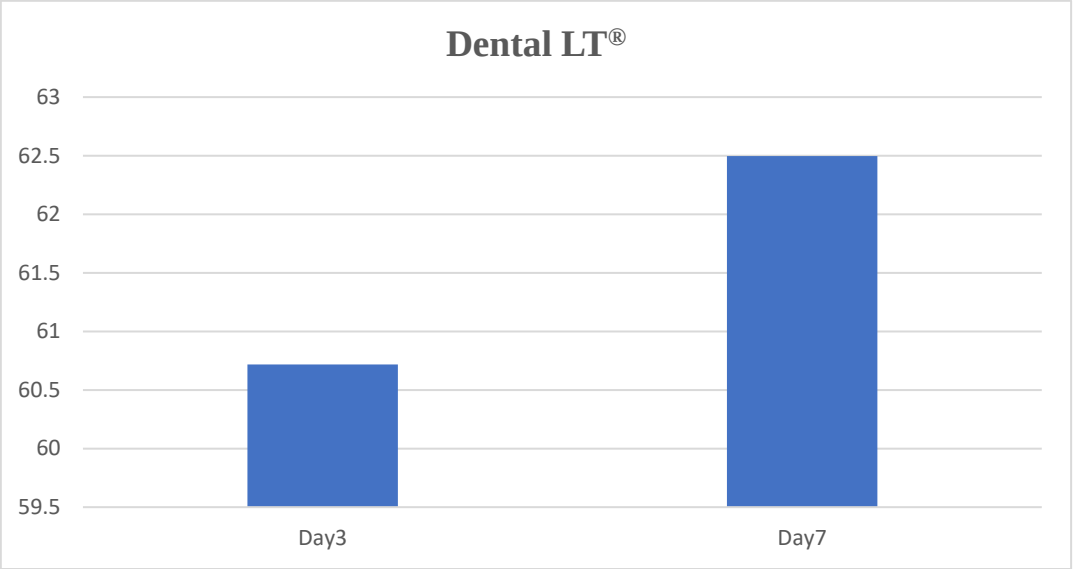
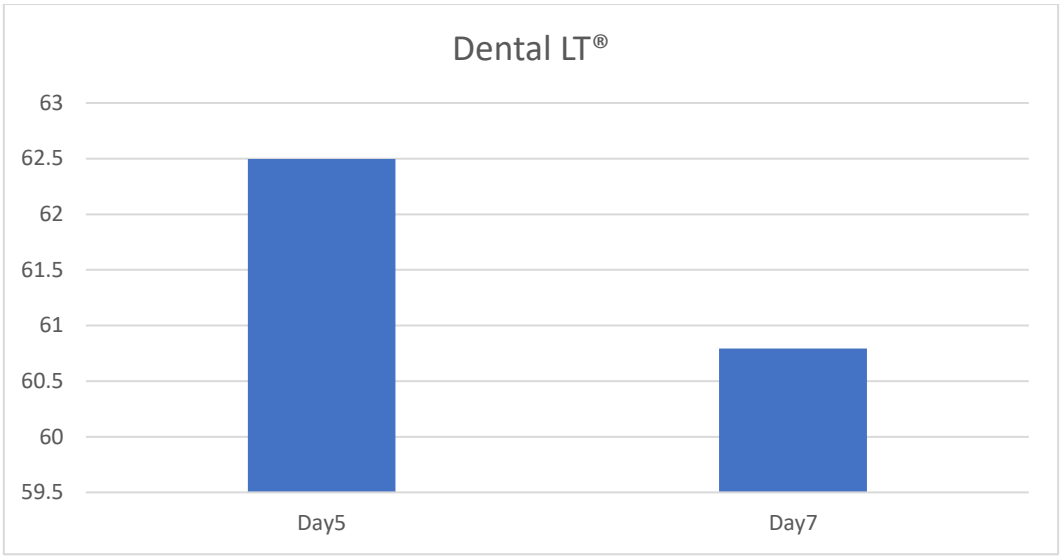


TABLE - 13

Day 5 vs Day 7											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	Sig.
					Lower Bound	Upper Bound					
Day 5	7	62.4977	2.5542	0.9654	60.1354	64.8599	59.01	66.67	-1.70465	1.6453	0.730
Day 7	7	60.793	3.15019	1.19066	57.8796	63.7065	56.94	64.72			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 12


Dental LT®
63
62.5
62
61.5
61
60.5
60
59.5
Day5
Day7

Comparison of Day 5 vs Day 7 (Dental LT®)

Comparison of cell viability of Dental LT® between Day 5 and Day 7 is given in Graph 12. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 5 and Day 7 analyzed with Tukey test is tabulated in Table 13. There is **no statistically significant** (P-value 0.05) difference in Day 5 and Day 7 cell viability of Dental LT®. The results indicate cell viability of day 7 sample to be less when compared to cell viability of day 5 sample. Methacrylate material seems to be more toxic on day 7 than day 5 but the difference is insignificant statistically.

For Dental LT® the cytotoxicity was comparatively more on day 1 and as days progressed, there was a reduction in cytotoxicity, but there was a slight increase in cytotoxicity on day 7 as shown in the above-mentioned tables and graphs. The differences in cytotoxicity in between days was not statistically significant.

Accura 60®

Comparison of Day 1 vs Day 3

Comparison of cell viability of Accura 60® between Day 1 and Day 3 is given in Graph 13. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 1 and Day 3 analyzed with Tukey test is tabulated in Table 14. There is **statistically significant** (P value<0.05) difference in Day 1 and Day 3 cell viability of Accura 60®. The results indicate cell viability of day 1 sample to be less when compared to cell viability of day 3 sample. Polycarbonate material seems to be more toxic on day 1 than day 3 as it is evident statistically.

TABLE - 14

Day 1 vs Day 3											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	P value
					Lower Bound	Upper Bound					
Day 1	7	13.915	3.8218	1.4445	10.3806	17.4497	10.64	21.79	-6.50361*	1.5994	0.002
Day 3	7	20.419	3.9595	1.4966	16.7568	24.0807	12.32	24.25			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 13

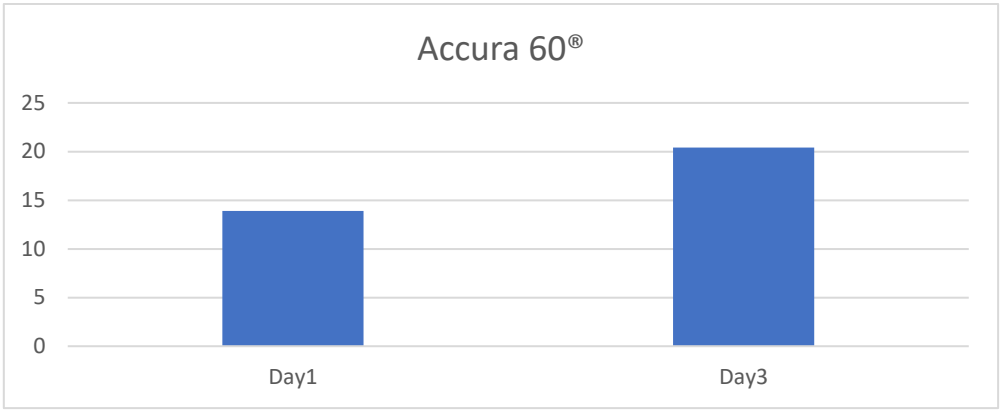
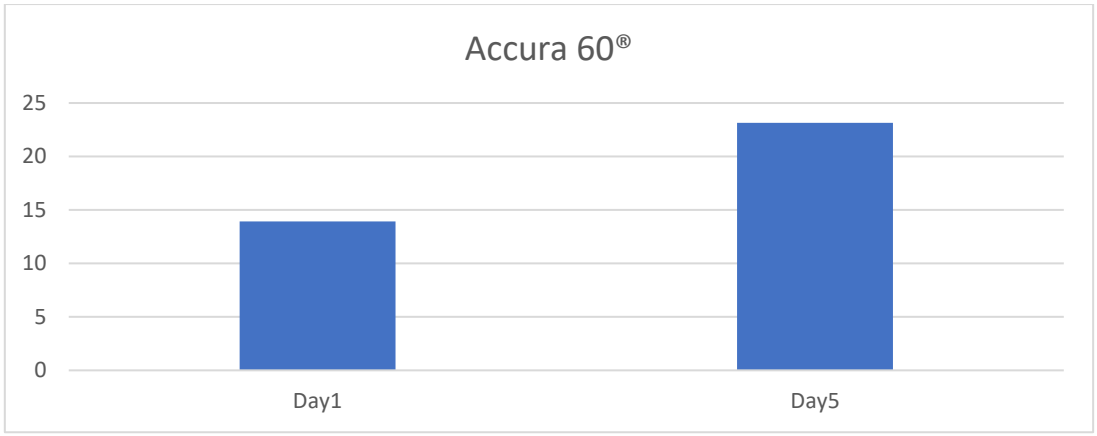


TABLE - 15

Day 1 vs Day 5											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	P value
					Lower Bound	Upper Bound					
Day 1	7	13.915	3.8218	1.4445	10.3806	17.4497	10.64	21.79	-9.24588*	1.5994	0
Day 5	7	23.161	1.8808	0.7109	21.4215	24.9005	19.2	24.9			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 14


Accura 60®
25
20
15
10
5
0
Day1
Day5

Comparison of Day 1 vs Day 5 (Accura 60®)

Comparison of cell viability of Accura 60® between Day 1 and Day 5 is given in Graph 14. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 1 and Day 5 analyzed with Tukey test is tabulated in Table 15. There is **statistically significant** (P value<0.05) difference in Day 1 and Day 5 cell viability of Accura 60®. The results indicate cell viability of day 1 sample to be less when compared to cell viability of day 5 sample. Polycarbonate material seems to be more toxic on day 1 than day 5 as it is evident statistically.

Comparison of Day 1 vs Day 7 (Accura 60®)

Comparison of cell viability of Accura 60® between Day 1 and Day 7 is given in Graph 15. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 1 and Day 7 analyzed with Tukey test is tabulated in Table 16. There is **statistically significant** (P value<0.05) difference in Day 1 and Day 7 cell viability of Accura 60®. The results indicate cell viability of day 1 sample to be less when compared to cell viability of day 7 sample. Polycarbonate material seems to be more toxic on day 1 than day 7 as it is evident statistically.

Comparison of Day 3 vs Day 5 (Accura 60®)

Comparison of cell viability of Accura 60® between Day 3 and Day 5 is given in Graph 16. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 3 and Day 5 analyzed with Tukey test is tabulated in Table 17. There is **no statistically significant** (P-value 0.05) difference in Day 3 and Day 5 cell viability of Accura 60®. The results indicate cell viability of day 3 sample to be less

when compared to cell viability of day 5 sample. Polycarbonate material seems to be more toxic on day 3 than day 5 but the difference is not significant statistically.

TABLE - 16

Day 1 vs Day 7											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	P value
					Lower Bound	Upper Bound					
Day 1	7	13.915	3.8218	1.4445	10.3806	17.4497	10.64	21.79	-10.59848*	1.5994	0
Day 7	7	24.514	1.4109	0.5333	23.2087	25.8185	21.66	25.81			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 15

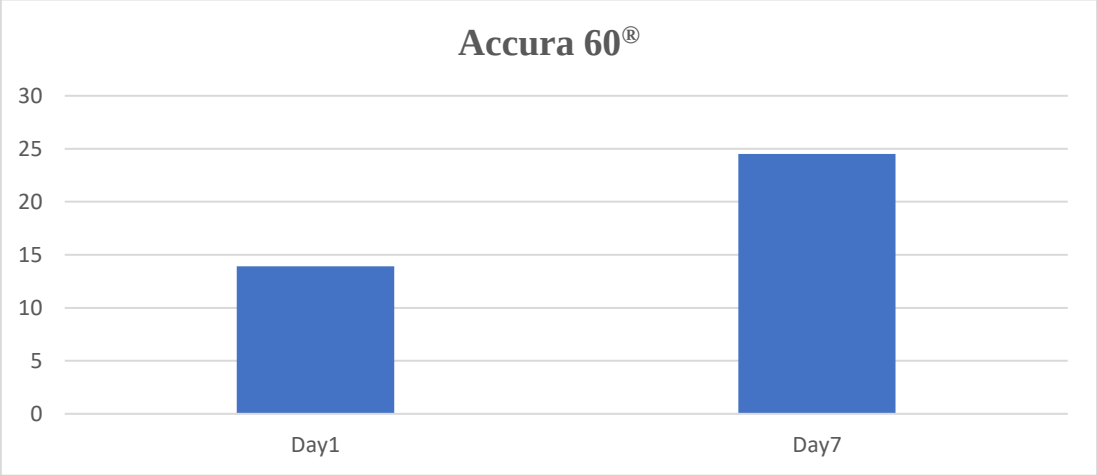


TABLE - 17

Day 3 vs Day 5											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	P value
					Lower Bound	Upper Bound					
Day 3	7	20.419	3.9595	1.4966	16.7568	24.0807	12.32	24.25	-2.74226	1.5994	0.338
Day 5	7	23.161	1.8808	0.7109	21.4215	24.9005	19.2	24.9			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 16

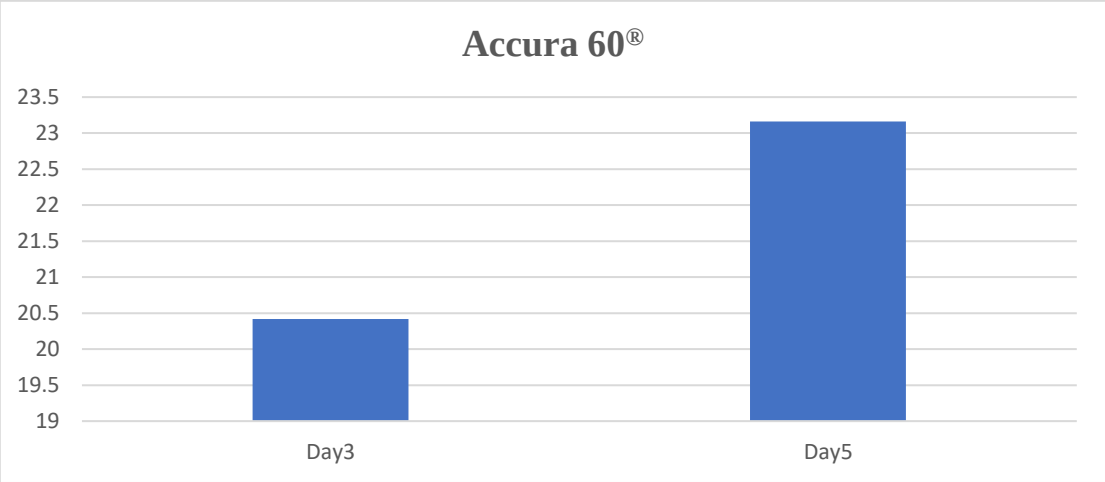


Table 18

Day 3 vs Day 7											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	P value
					Lower Bound	Upper Bound					
Day 3	7	20.419	3.9595	1.4966	16.7568	24.0807	12.32	24.25	-4.09487	1.5994	0.076
Day 7	7	24.514	1.4109	0.5333	23.2087	25.8185	21.66	25.81			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 17

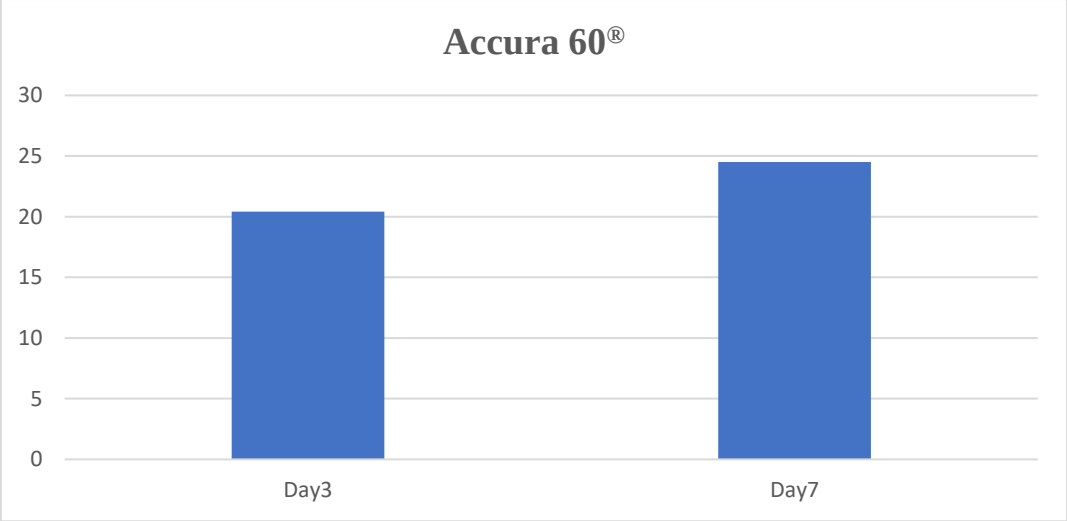
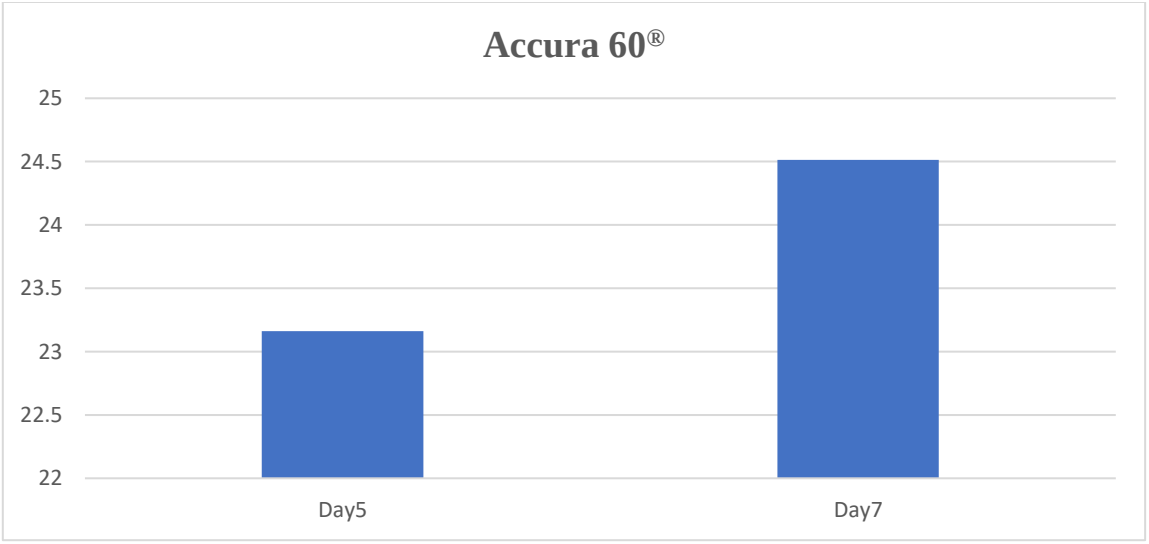

Accura 60®
30
25
20
15
10
5
0
Day3
Day7

TABLE - 19

Day 5 vs Day 7											
Cell viability % Tukey HSD											
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Mean Difference (I-J)	Std. Error	P value
					Lower Bound	Upper Bound					
Day 5	7	23.161	1.8808	0.7109	21.4215	24.9005	19.2	24.9	-1.3526	1.5994	0.832
Day 7	7	24.514	1.4109	0.5333	23.2087	25.8185	21.66	25.81			

P value < 0.05- S, P value > 0.05- NS

GRAPH - 18



Comparison of Day 3 vs Day 7 (Accura 60®)

Comparison of cell viability of Accura 60® between Day 3 and Day 7 is given in Graph 17. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 3 and Day 7 analyzed with Tukey test is tabulated in Table18. There is **no statistically significant** (P-value 0.05) difference in Day 3 and Day 7 cell viability of Accura 60®. The results indicate cell viability of day 3 sample to be less when compared to cell viability of day 7 sample. Polycarbonate material seems to be more toxic on day 3 than day 7 but the difference is not significant statistically.

Comparison of Day 5 vs Day 7 (Accura 60®)

Comparison of cell viability of Accura 60® between Day 5 and Day 7 is given in Graph 18. The mean, standard deviation, standard error and 95% confidence interval, mean difference and P value for Day 5 and Day 7 analyzed with Tukey test is tabulated in Table 19. There is **no statistically significant** (P-value 0.05) difference in Day 5 and Day 7 cell viability of Accura 60®. The results indicate cell viability of day 5 sample to be less when compared to cell viability of day 7 sample. Polycarbonate material seems to be more toxic on day 5 than day 7 but the difference is not significant statistically.

For Accura 60® material the cytotoxicity was comparatively more on day 1 and as days progressed, there was a reduction in cytotoxicity as shown in the above-mentioned tables and graphs. The differences were statistically significant for day 1 when compared to all other days, but the differences were not statistically significant between day 3, day 5 and day 7.

Intergroup findings

The mean, standard deviation, standard error and 95% confidence interval for inter group findings are calculated and tabulated. One-way ANOVA (Table 20) was done to compare all three materials. Tukey test was done to compare any two groups. There were **statistically significant** (P value<0.05) differences in cell viability between 3 material groups. Inter-group findings were analyzed as follows

Intergroup comparison for Day 1

Intergroup differences in cell viability between 3 materials (Invisalign®, Dental LT® and Accura 60®) for day 1 were analyzed using Tukey test (Table 21). There was a **statistically significant** difference (P value < 0.05) in cell viability % of each of these materials on day 1. Mean cell viability values for these 3 materials on day 1 is given in Graph 19. Day 1 samples were more cytotoxic for all 3 materials than the consecutive days, but on day 1 Invisalign® had lesser cytotoxicity when compared to Dental LT® and Accura 60® as it is evident statistically. Accura 60® was more cytotoxic when compared to Dental LT®.

Intergroup comparison for Day 3

Intergroup differences in cell viability between 3 materials (Invisalign®, Dental LT® and Accura 60®) for day 3 are analyzed using Tukey test (Table 22). There was a **statistically significant** difference (P value < 0.05) in cell viability % of each of these materials (M1, M2 and M3) on day 3. Mean cell viability values for these 3 materials on day 3 is given in Graph 20. On day 3 Invisalign® had lesser cytotoxicity when compared to Dental LT® and Accura 60® as it is evident statistically. Accura 60® was more cytotoxic when compared to Dental LT®.

Intergroup comparison for Day 5

Intergroup differences in cell viability between 3 materials (Invisalign®, Dental LT® and Accura 60®) for day 5 were analyzed using Tukey test (Table 23). There was a **statistically significant** difference (P value < 0.05) in cell viability % of each of these materials on day 5. Mean cell viability values for these 3 materials on day 5 is given in Graph 21. On day 5 Invisalign® had lesser cytotoxicity when compared to Dental LT® and Accura 60® as it is evident statistically. Accura 60® was more cytotoxic when compared to Dental LT®.

TABLE 20- ANOVA

Cell viability %

Day		Sum of Squares	Df	Mean Square	F	P value
Day1	Between Groups	14226.111	2	7113.056	483.400	.000
	Within Groups	264.863	18	14.715		
	Total	14490.975	20			
Day3	Between Groups	11550.589	2	5775.295	265.838	.000
	Within Groups	391.047	18	21.725		
	Total	11941.636	20			
Day5	Between Groups	10324.948	2	5162.474	249.946	.000
	Within Groups	371.778	18	20.654		
	Total	10696.726	20			
Day7	Between Groups	9528.694	2	4764.347	606.718	.000
	Within Groups	141.348	18	7.853		

TABLE 20- ANOVA

Cell viability %

Day		Sum of Squares	Df	Mean Square	F	P value
Day1	Between Groups	14226.111	2	7113.056	483.400	.000
	Within Groups	264.863	18	14.715		
	Total	14490.975	20			
Day3	Between Groups	11550.589	2	5775.295	265.838	.000
	Within Groups	391.047	18	21.725		
	Total	11941.636	20			
Day5	Between Groups	10324.948	2	5162.474	249.946	.000
	Within Groups	371.778	18	20.654		
	Total	10696.726	20			
Day7	Between Groups	9528.694	2	4764.347	606.718	.000
	Within Groups	141.348	18	7.853		
	Total	9670.042	20			

P value < 0.05- S, P value > 0.05- NS

TABLE 21 - DAY 1 TUKEY HSD

Material	N	Subset for alpha = 0.05		
		1	2	3
Accura 60®	7	13.9151		
Dental LT®	7		60.2557	
Invisalign®	7			75.0046

TABLE 21 - DAY 1 TUKEY HSD

Material	N	Subset for alpha = 0.05		
		1	2	3
Accura 60®	7	13.9151		
Dental LT®	7		60.2557	
Invisalign®	7			75.0046
P value		0.000	0.000	0.000

P value < 0.05- S, P value > 0.05- NS

GRAPH - 19

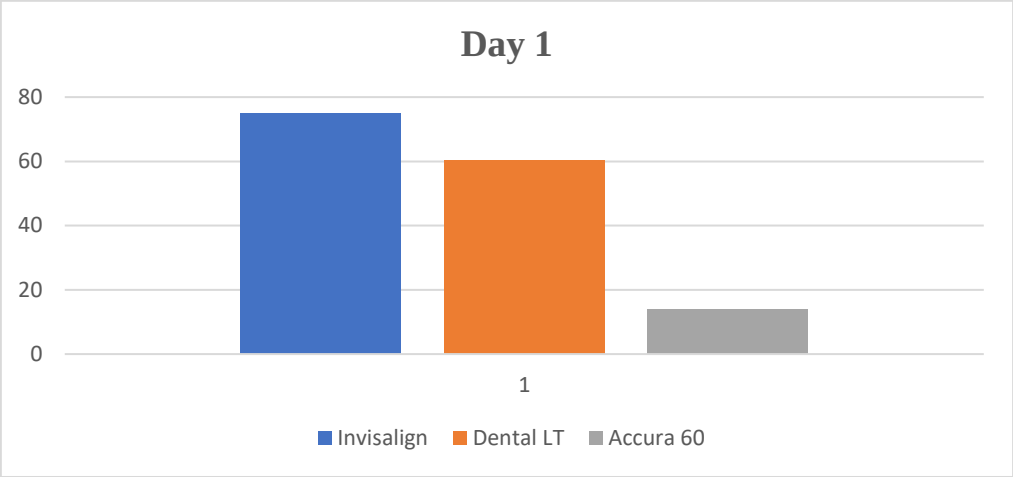


Table 22 Day=Day3 Tukey HSD

Material	N	Subset for alpha = 0.05		
		1	2	3
Accura 60®	7	20.4188		
Dental LT®	7		60.7189	
Invisalign®	7			76.0237
P value		0.000	0.000	0.000

P value < 0.05- S, P value > 0.05- NS

GRAPH - 20

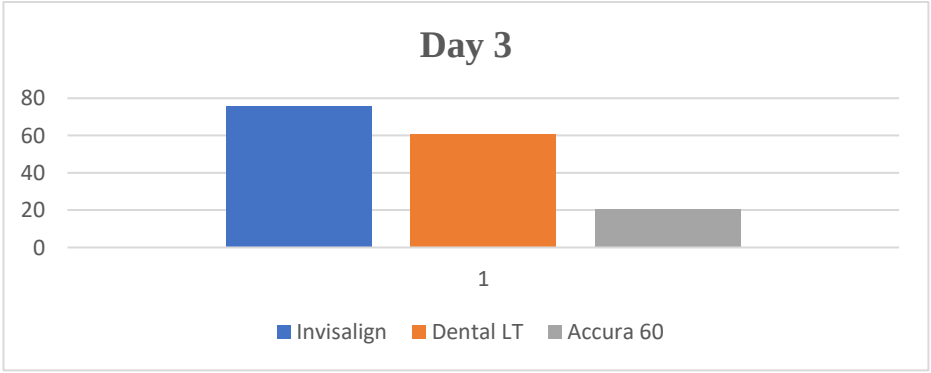
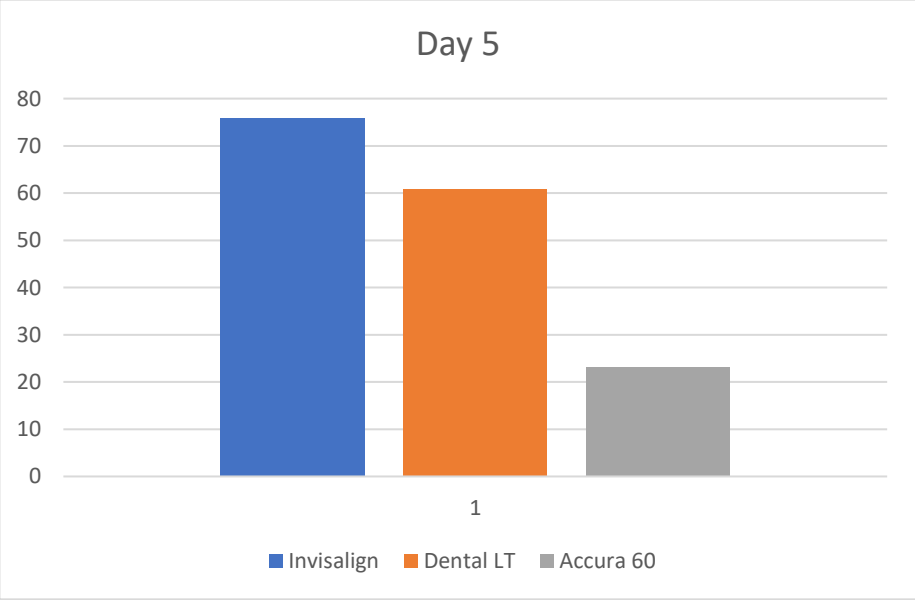


TABLE - 23 Day=Day5 Tukey HSD

Material	N	Subset for alpha = 0.05		
		1	2	3
Accura 60®	7	23.1610		
Dental LT®	7		60.7930	
Invisalign®	7			75.8940
P value		0.000	0.000	0.000

P value < 0.05- S, P value > 0.05- NS

GRAPH - 21



Intergroup comparison for Day 7

Intergroup differences in cell viability between 3 materials (Invisalign®, Dental LT® and Accura 60®) for day 1 are analyzed using Tukey test (Table 24). There was a **statistically significant** difference (P value < 0.05) in cell viability % of each of these materials on day 7. Mean cell viability values for these 3 materials on day 7 is given in Graph 22. On day 7 Invisalign® had lesser cytotoxicity when compared to Dental LT® and Accura 60® as it is evident statistically. Accura 60® was more cytotoxic when compared to Dental LT®.

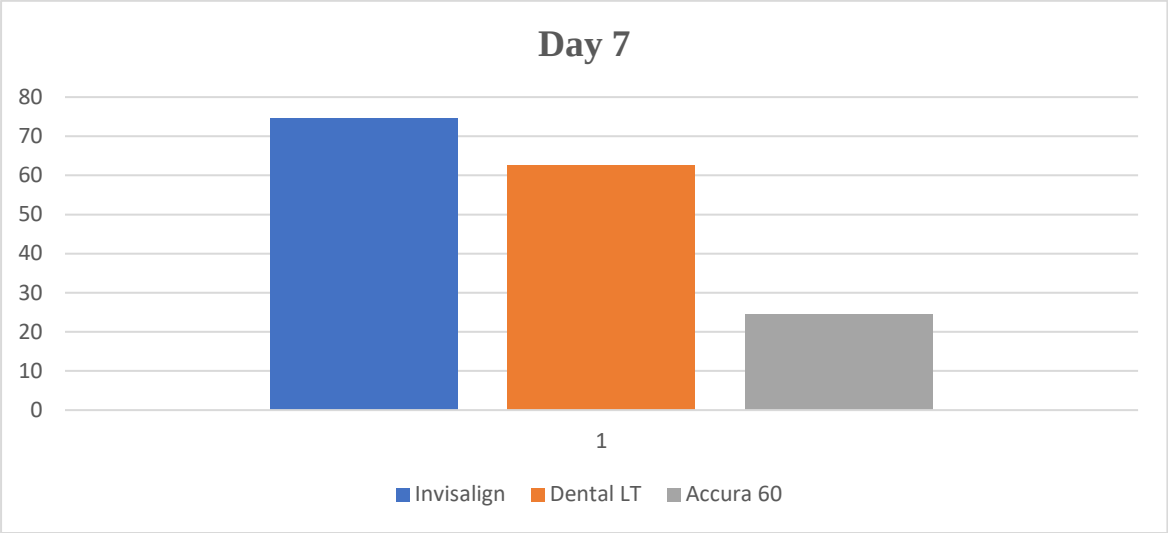
Table 24 Day=Day7

Tukey HSD

Material	N	Subset for alpha = 0.05		
		1	2	3
Accura 60®	7	24.5136		
Dental LT®	7		62.4977	
Invisalign®	7			74.4858
P value		0.000	0.000	0.000

P value < 0.05- S, P value > 0.05- NS

GRAPH - 22



DISCUSSION

Esthetics has been the main concern for patients undergoing orthodontic treatment. Adults and professionals nowadays prefer an esthetic alternative to conventional labial fixed appliances. This ever-growing emphasis on esthetics and new technologies had led to the introduction of various esthetic alternatives to treating malocclusion. Invisalign® is a stereolithographic clear aligner made of polyurethane[6] which have been in use for the past 2 decades in treating malocclusion. Also, there are several other esthetic clear aligner systems in use at present for treating malocclusion. Polycarbonate brackets [(10)] and arch wires provide an esthetic alternative to fixed labial orthodontics. Recently Dental LT®, a methacrylate-based stereolithographic photo polymeric resin introduced by Form labs® which they claim to be a class IIa biocompatible material.

Invisalign® is polyurethane based material. Polyurethane is a polymer of 4,4[l] di-methyl diisocyanate and leaching of this causes cytotoxicity, but saliva acts as a buffer from the cytotoxic effects of isocyanate from Invisalign® tray.[6] Polycarbonate is known for leaching of bisphenol- A [26] which is highly cytotoxic. Previous literature suggests leaching of cytotoxic methacrylate monomer from various methacrylate polymeric products.[21] Leaching of such cytotoxic chemicals makes us question the biocompatibility of these materials and its safety in day to day intraoral usage.

There have been several cytotoxic studies done to check biocompatibility of various orthodontic materials for the past 2 decades. There are already a few studies carried out to check the cytotoxicity of various clear aligner systems and most of them deduct that they have statistically insignificant levels of cytotoxicity and are safe for intraoral usage.[12] There has been no previous study done on cytotoxicity of Dental LT®

though it has been used for fabrication of deprogramming hard splint. Also, there are several cytotoxicity studies in the literature assessing polycarbonate brackets and arch wires.[22] With an increasing need for these esthetic clear aligners, esthetic brackets and 3D printed auxiliaries for long-term intraoral usage, assessing their cytotoxicity and determining if the material is biocompatible is of prime need.

Various cell characteristics and functions are used to investigate the cytotoxicity of medical devices. Some researchers have evaluated the cell viability, adhesion, proliferation, and metabolism of cells such as 3T3[10], L929[22], and W138[22], and human fibroblasts[6,9,11,12,20] and osteoblasts. In this study, 3T3 embryonic mouse fibroblast cell line was used for studying cell viability.

MTT assay which is most preferred and easily available cytotoxicity assay for medical instruments, equipment and drugs.[7] MTT is a tetrazolium Bromide reduction assay. Its mechanism is, healthy viable cells with active metabolism convert MTT into a purple colored formazon product with an absorbance maximum near 570 nm.[7] Formazon crystals precipitate in the cell culture medium solution and it must be dissolved before measuring optical density. For this purpose, dimethyl sulfoxide[7] (DMSO) is used. It dissolves the water-insoluble formazon crystals to form purple colored liquid. Greater the change in color, greater is the proportion of healthy cells. the change in color is quantified by optic density of the solution after 5 hour incubation period[8] after the addition of MTT. Measuring the optical density was done with the help of ELISA reader.

With the interest in assessing the biocompatibility of these newer esthetic plastic materials for long term intraoral usage, this study has been undertaken. In this prospective study, cytotoxicity of stereolithographic printed 3D splints were evaluated as it will open newer possibilities for its usage in digital orthodontics.

In my study, the intragroup findings of cell viability for polyurethane (Invisalign®) when compared to control was found to be less viable, indicating that material at the end of day 1 has resulted in some amount of toxicity, due to the leaching of di-isocyanate[31]. The cell viability values slightly increased for days 3, 5 and 7 respectively but the increase in cell viability was not statistically significant, indicating that as the days progressed the cytotoxicity reduced. Similar to my study, cytotoxicity of polyurethane sample was done by **Kotyk et al**[32], for 1, 3 and 7 days. Findings of the study indicate leaching of diisocyanate was more on day 1 and as the days progressed the leaching decreased, so the cytotoxicity decreased and reversing the cell viability. Various other in-vitro cytotoxicity studies done for polyurethane shows that the cytotoxicity for day 7 was minimal, which was similar to the results of my study. **Premaraj et al** [6] suggested that the reason for the decrease in toxicity and increase in cell viability could be due to the presence of a tenacious layer of saliva over the tissues to be an important factor for preventing the diisocyanate from acting on the cells.

In my study, the intragroup findings of cell viability for methacrylate (Dental LT®) when compared to control was found to be less cell viable, indicating that material at the end of day 1 has resulted in some amount of toxicity, due to the leaching of methacrylate monomer.[21,33] The differences in cell viability were not statistically significant, indicating that the material toxicity decreased from day 1 to day 5 but there was slight increase in toxicity on day 7. Similar to my study cytotoxicity of methacrylate sample was done by Kopperud et al[23], **Ahrari et al**[20] and **Ozturk et al**[9] for 1, 3, 5 and 7 days. The findings of these studies indicate the leaching of methacrylate was more on day 1 and as days progressed the leaching decreased, reversing the cell viability. According to Kopperud **et**

al[23]**,** the cytotoxic effect of methacrylate is due to genotoxicity of methacrylate monomer whereby it directly affects the DNA by the formation of reactive oxygen species.

The intragroup findings of cell viability for polycarbonate (Accura 60®), when compared to control was found to be less viable, indicating that material at the end of day 1 the material was cytotoxic. This is due to the leaching of Bisphenol A. The cell viability values increased for days 3, 5 and 7 respectively and the increase in cell viability values were statistically significant, indicating that as the days progressed the cytotoxicity reduced. Similar to my study cytotoxicity of polycarbonate sample was done by **Retamoso et al**[10]**, Vitral et al**[22]**, Kotyk et al** [32] and **Hanshella et al**[34] for 1, 3 and 7 days. The findings of these studies indicate the leaching of Bisphenol A was more on day 1 and as days progressed the leaching decreased, reversing the cell viability. According to **Terasaka et al**[17], Bisphenol A is an estrogen-like substance, and it causes cytotoxicity by activation of mitochondrial apoptosis through its action on genes (AIF, cytochrome c and SMAC/ Diablo)[30] which reduce the anti-apoptotic factor.

The **intergroup findings** for cell viability for three tested samples at the end of **day 1, day 3, day 5 and day 7,** indicates that **polyurethane (Invisalign®)** had significantly **better cell viability** values when compared to other two samples. Polycarbonate had less cell viability (i.e. more toxicity) when compared to polyurethane and methacrylate oligomer - glycol methacrylate for all the days. The differences in cell viability between all 3 test samples (Invisalign®, Dental LT® and Accura 60®) were statistically significant. This indicate that polyurethane (Invisalign®) was the least cytotoxic followed by Methacrylate oligomer - glycol methacrylate (Dental LT®) and polycarbonate (Accura 60®). Polycarbonate was the most toxic of the three tested samples. The intergroup findings of my study for increase in toxicity of polycarbonate at the day 1, 3, 5 and 7 may be due to increased leaching associated with it.

The increased leaching associated with polycarbonate in our study was similar to the study done by **Vitral et al**[22]**,** where they tested polycarbonate leaching for day 1, 2 and 3 and concurred the leaching is more between 24 to 48 hours. In a study done by **Kotyk et al**[32] where they compared the leaching of polyurethane and polycarbonate it, was found that increased leaching associated with polycarbonate. The result of my study was **similar** to the study done by **Kopperud et al** [23] in which he compared the quantity of leaching from polyurethane and methacrylate on days 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 and found more leaching associated with methacrylate when compared to polyurethane.

The limitation of this study includes cell viability was assessed only for a shorter period of time (i.e. 1 – 7 days). But this 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.[22,30,32] Long term studies which have evaluated the cytotoxicity have concluded that changes in cytotoxicity were severe in first few days and there was no significant increase in cell viability after first one week of evaluation.

Future studies should focus on Selective Laser Sintering (SLS) metal 3D printing materials for ion leaching and assess their cytotoxicity for intraoral biocompatibility in orthodontic usage. Also, in-vitro studies should be done for cytotoxic evaluation of 3D printing material using human cells to evaluate its safer intraoral usage.

SUMMARY

With the increase in popularity of clear aligners and newer materials for 3D printed appliances, it becomes necessary to assess the cytotoxicity of these materials which have an end usage intraorally. In this perspective, cytotoxicity of three different 3D printed materials (Invisalign®, Dental LT® and Accura 60®) using MTT assay was evaluated in-vitro for a period of 1, 3, 5 and 7 days. The sample materials were 3D printed using a standardized procedure with stereolithography apparatus (SLA). The cytotoxicity study conducted on 3T3 embryonic mice fibroblast cell line evaluated the cell viability in relation to the SLA 3D materials. Cytotoxic assessment of these samples were according to international organization for standardization (ISO 10993) norms. Using MTT assay, the cell viability % was assessed for each sample material.

Further optical density was used to measure each sample using ELISA plate reader and this was used in assessing cytotoxicity. Cell viability values for polyurethane (Invisalign®), methacrylate (Dental LT®) and polycarbonate (Accura 60®) were obtained and assessed statistically using one-way ANOVA and Tukey's test. Significant differences were found in cell viability values of all three materials. Results suggested that polyurethane was the least toxic followed by methacrylate oligomer- glycol methacrylate and polycarbonate.

Within the limits of this study, polyurethane (Invisalign®) and methacrylate oligomer – glycol methacrylate (Dental LT®) were biocompatible and safe for intraoral orthodontic usage. Polycarbonate (Accura 60®) was significantly more toxic than polyurethane and methacrylate oligomer – glycol methacrylate and its usage intraorally is questionable.

CONCLUSION

- Stereolithography 3D materials are revolutionizing orthodontics with precise patient-specific appliances.
- Invisalign® material (polyurethane) was found to be more biocompatible than the other stereolithographic materials.
- Cytotoxicity was found to be more on the first day and gradually decreases as days progress. This indicates increased leaching of material during the initial period of use.
- Accura 60® material showed increased in-vitro cell death suggesting that it is non-biocompatible due to its increased cytotoxicity.
- Invisalign® and Dental LT® materials are safer for intraoral orthodontic usage as both the material are biocompatible.

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