ORAL EPITHELIAL CELL REACTION AFTER EXPOSURE TO ESSIX AND HAWLEY'S RETAINERS: AN IN- VITRO COMPARATIVE STUDY

By

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ABSTRACT

Background and Objectives: Removable retainers are often used after fixed appliance therapy to hold the teeth in their corrected positions. Hawley's and Essix retainer are the most commonly prescribed appliances for retention. Hawley's retainer can be fabricated with self cure or heat cure acrylic resin, among these self cure acrylic is the most commonly used in orthodontics. The Essix retainer which is fabricated with polyurethane plastic has gained popularity among orthodontic patients as these are almost invisible and hence more esthetic. As these retention appliances are worn for a longer period of time, the potential effects of these materials on the oral epithelium and their biocompatibility is very crucial. The purpose of this study was to evaluate the cytotoxicity of Essix and Hawley's retainer (self cure) over a long period of time on an in-vitro environment.

Methods: Eluate of cold cure acrylic resin and Essix retainer material was obtained by soaking 0.1g of particulated material in 1ml of artificial saliva. The artificial saliva served as the control group. The eluates of Cold cure acrylic and Essix retainer were tested at different time intervals-1 month, 2 months, 4 months, 6 months, 8 months and 1 year for cytotoxicity. The cytotoxicity testing was done on L929 Fibroblast cell lines using MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide) assay. The Optical density values were obtained at two different wavelengths to avoid background noise. Percentage of viable cells were calculated from the optical density values using a formula.

Results: Comparison of the cell viability among different groups at different time intervals showed a significant (p value<0.05) difference in the 8 months and 1 year samples of Essix retainer. Comparison of the cell viability values within the Essix retainer group showed statistically significant difference with a p value of 0.03.

Interpretation and conclusion: Although statistically significant differences were found when comparing different groups, the percentage cell viability values of the groups were within the normal limit. Those samples with more than 100 % values in MTT assay that indicates cell proliferation cannot be confirmatory as it can be due to multiple factors. A second cytotoxicity test, preferably a real time analysis is required to confirm if the cell proliferation observed is significant.

Keywords: Essix retainer, Hawley's retainer, MTT assay.

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INTRODUCTION

INTRODUCTION

Moyers defined retention as "The holding of teeth following orthodontic treatment in the treated position for the period of time necessary for the maintenance of the results."[1] The success of any orthodontic treatment depends on maintaining the teeth in the corrected position after debonding. Retention appliances are used after fixed appliance therapy for this purpose. The term "relapse" has been defined as the regression to the original malocclusion after orthodontic correction. But any change from the final position of teeth can be considered as relapse. Approximately 70% of the cases have a tendency for relapse after orthodontic treatment. This makes retention a very crucial subject following fixed orthodontic therapy.[4]

After the correction phase, a retention phase is integrated into orthodontic therapy to maintain the results for a longer period of time. This phase of treatment is accomplished by the use of fixed or removable retainers. The removable retainers have many advantages over the fixed retainers which makes it the most commonly prescribed retention appliance. Hawley's and Vacuum Formed Retainers (VFRs) are the two commonly used removable retention appliances.

The Hawley's retainer was introduced in 1919 by Charles Hawley.[2] It can be fabricated using either heat cure or cold cure acrylic resin which is composed of polymethyl methacrylate. The appliance comprises of a labial bow and 2 Adams clasps integrated into an acrylic baseplate. The labial bow covers the upper anteriors and effectively holds the anterior teeth in position. With relevant modifications, the Hawley's appliance can also close the band spaces, residual extraction spaces, control the incisor torque and allow settling of posterior occlusion.

In 1980s, vacuum formed clear thermoplastic sheets fitting snugly over the teeth were introduced into orthodontics as "clear retainers".[3] These retainers are commercially named as Essix retainers. Essix retainer is an aesthetic, comfortable and inexpensive modern alternative to traditional retainers. It is an almost invisible plastic device made from polyurethane plastic which is the same material used in invisalign appliances. However, in contrary to the Invisalign appliances which are meant to be used full time for a maximum of 2 weeks, the Essix retainers are worn for a prolonged duration, mostly on a part time basis. The Essix appliance completely encapsulates the

dentition and the superior part of the alveolus and thus provides better retention. The advantages of these retainers are aesthetics, low cost and simple fabrication technique. Breakage, occlusal wear and limited vertical settling of teeth are among the disadvantages of Vacuum Formed Retainers (VFR). Furthermore, this retainer is not as effective as Hawley's retainer in preventing bite deepening.[4]

The main disadvantage associated with a Hawley's retainer is the display of wires on the labial surface of teeth which is unaesthetic. VFRs have greater acceptance among patients because of many reasons such as esthetics, ease of maintenance, reduced fabrication time and cost. It can also be modified to produce minor tooth movements and can be used as carriers for bleaching solutions. Currently, both Essix and Hawley's retainers are frequently used in orthodontic practice.

There are different opinions regarding the suitable protocol for the wear of orthodontic retainers. According to Proffit et al, Hawley's retainers should be worn full-time for 3-4 months, and then night-only for at least 1 year following active orthodontic treatment. In comparison, the suggested protocol for vacuum-formed retainers is all-time wear for 1 week and then night-time only (8 hours a day) for at least 1 year. Since the remodeling of periodontal fibers occurs during the first 3-4 months after appliance removal, full-time wear of VFRs for just 1 week seems to be not effective in preventing relapse in orthodontic patients.[4]

The unease about the release of chemicals from various appliances has grown over recent decades. Certain studies have indicated the release of Bisphenol A (BPA) from Essix retainers when used intraorally. BPA is a synthetic compound that has gained medical attention because of its estrogenic action. In 2011, the World Health Organization (WHO) has listed BPA as an endocrine disruptive chemical.

Acrylic resin which is used in the fabrication of Hawley's retainer is composed of high molecular weight polymers which polymerizes in an addition reaction. There are thermo polymerized and autopolymerized acrylic resins. Among these, the autopolymerized resins remain the most popular material for use in orthodontics because of their low cost and ease of use.[5] The polymerization of acrylic resin is very critical for the optimization of the material's physical and biological properties because it facilitates the conversion of monomers into polymers. If the polymerization is incomplete it can lead to leaching out of residual monomers or

produce toxic chemical products such as MMA(methyl methacrylate), formaldehyde, methacrylic acid, benzoic acid, dibutylphthalate, phenyl benzoate and phenyl salicylate.[6] Such harmful chemicals when released into oral environment can trigger hypersensitivity and allergic reactions which can later lead to systemic involvement.

As the Essix retainer is fabricated from a thermoplastic resin by a thermoforming process, one serious concern regarding it is that plasticized materials can leach out chemical substances called xenoestrogens into the immediate environment surrounding the plastic. These substances have the ability to produce a biological reaction comparable to that of estrogen hormones, which are capable of inducing estrogenic signals that can modify gene expression. [7-9] One of the materials concerned is BPA, an important starting material for the production of epoxy resins and polycarbonates, which is manufactured by acid catalysed condensation of acetone and phenol.[10,11]

The accumulated level of BPA in the body may vary according to the developmental stage and gender of the subject. According to the United States Environmental Protection Agency (USEPA), and the Food and Drug Administration Agency (FDA), the acceptable daily intake dose is 50 μg/ kg/day of BPA. However, adverse effects have been documented with BPA doses below the above-mentioned daily levels also. So the safety and biological nature of such thermoplastic material is very crucial for its clinical use. With continued use, these resins are supposed to release toxic substances like methylene diphenyldiisocyanate (MID) and 1,6-hexanediol diacrylate (HD). These chemicals have a melting point of about 37-39°C and 5°C, respectively.[12] These plastic materials can also be affected by changes in the oral environment and then release molecules that could be dangerous to oral cells. For these reasons, the cytotoxicity of Essix retainer materials need to be investigated.[13]

The potential ranges of cytotoxic effects from these retainers can be many because of their extended use which could cause degradation of the material. The cytotoxic effects includes an immune reaction to material exposure, cell cycle disturbance, cell apoptosis and induction of mutagenesis or carcinogenesis. These effects are not always seen immediately.[14] The Essix retainer being a polyurethane plastic material, it's Isocyanate composition might pose health issues. Isocyanate

usually causes irritations on mucous membrane. It can also cause asthmatic or hypersensitivity reactions depending on the duration of exposure. Loss of membrane integrity due to epithelial cell death or damage to the epithelial layer could lead to hexamethylene diisocyanate conjugated protein exposure to the human immune system.[15,16] As a result, it could trigger immunologic reactions.

Testing of cytotoxic effects of dental materials in vitro on cell lines by cell culture methods is relatively easy, reproducible and cost-effective and can be carefully controlled. These tests are more appropriate than animal experiments, which can introduce uncontrolled variables.

In this study, we investigated the oral epithelial cell reaction to Essix and Hawley's retainers. Responses of cells through cell viability, changes in cell morphology and cell behaviour were assessed.

The methods for cytotoxicity analysis are described and regulated by ISO-standard 10993-5.[17, 18, 19] Numerous in-vitro assays are based on the measurement of cell viability and proliferation to know the response of a cell population to some treatment conditions.

MTT assay is one of the most sensitive tests for cytotoxicity testing. This method uses colorimeter to determine the cell viability (Mosmann et al.1983). In the absence of any cells, the MTT reagent shows low background absorbance values. A linear relationship exists between metabolically active cells and the colour produced. This allows to accurately quantify changes in rate of cell death or cell proliferation. (Van de Loosdrecht et al. 1994). MTT is the commonly applied method for evaluation of cell viability and cytotoxicity for screening drugs. [20]

The MTT assay is based on the reduction of MTT (yellow colored) and other tetrazolium dyes depends upon cellular metabolic activities due to NADPH-dependent cellular oxidoreductase enzymes into insoluble (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan (formazan).The healthy and rapidly growing cells reduces MTT to formazan at higher rates. The dead cells fail to reduce MTT. The final product after the reduction of MTT reagent is a purple coloured formazan crystal. These crystals can be easily dissolved in Dimethyl Sulfoxide (DMSO).[20] In this assay,

the viability is assessed by the quantification of the purple coloured formazan at 490 nm.

The intensity of the colour is measured in terms of optical density. It is linearly associated with the enzyme activity and is indirectly linked to the number of viable cells. A high optical density value indicates increased colour intensity which is associated with higher cell viability. A reduced intensity of the purple colour signifies some cell death and the cytotoxicity of the given substance.

Bural et al. reported a classification based on the cytotoxicity degree of the tested material as follows: noncytotoxic (cell proliferation greater than 75%), slight cytotoxicity (50–75% of cell proliferation), moderate cytotoxicity (25–50% of cell proliferation) and high cytotoxicity (cell proliferation less than 25%). [18]

Although in-vitro and in-vivo studies have been performed to assess BPA release from Hawley's and Essix retainer, there are no much studies on the cytotoxicity of Essix and Hawley's retainers. The biocompatibility of these materials should be tested as these appliances are used routinely in our practice. Cold cure acrylic material was chosen because it is the most commonly used in orthodontic retainers. Few studies have evaluated the biocompatibility of retainers. The invisalign appliances which is produced by the same thermoplastic material as Essix retainer have been investigated on cytotoxicity in a few studies. But unlike from the Invisalign appliance, the Essix retainers are worn for extended duration which might influence the biocompatibility.

OBJECTIVES

OBJECTIVES

1) To measure the optical density values at two wavelengths after Essix and cold cure materials are added to the microplate wells seeded with L929 fibroblast cells.
2) To calculate the percentage cell viability at different time intervals of both the Essix and Hawley's retainer samples and the controls from the obtained optical density values.
3) To estimate the cytotoxicity of the Hawley's and Essix retainer samples at different time intervals.
4) To compare the biocompatibility of Hawley's and Essix retainer at different time intervals based on the percentage of cell viability.

BACKGROUND & REVIEW OF LITERATURE

BACKGROUND OF THE STUDY

Appropriate retention protocol should be followed in every patient after fixed orthodontic treatment as the results obtained may be unstable due to the fact that the periodontal ligament fibers reorganize over a period of time. Three main types of retainers used are the Hawley's and Vaccum Formed removable retainers and the Fixed retainers. The Hawley's retention appliance is made of thermopolymerized or autopolymerized acrylic resin among which the autopolymerized acrylic is the most preferred among orthodontists. The Essix retainer is made of Polyurethane plastic which is the same material used in the fabrication of Invisalign appliances. The cytotoxicity of these retention appliances are a matter of concern as they remain in contact with the oral mucosa for an extended duration of time.

REVIEW OF LITERATURE

E. O. Dillingham et al (1975)[21] have done a biological evaluation of Polymethyl methacrylate. It was concluded that the observed variation of biological test results reflected significant differences in the toxicity of the test materials. The polymethyl methacrylate series examined was relatively low in toxicity and the biological tests examined, particularly the in vitro tests, were found to be responsive to formulation and curing conditions which indicated their suitability for primary toxicity screening.

Giunta J et al (1976)[22] investigated on allergic stomatitis caused by self polymerizing resin and reported that the allergen was monomer and they indicated methods of processing the self-polymerizing resin to allow it to become essentially non-reactive in a sensitized patient.

Hensten- Pettersen A et al (1981)[23] studied the cytotoxic potential of autopolymerized pour and dough type resins and heat cured resins in in-vitro cell culture. Human epithelial cells (NCTC 2544) were grown in Eagle's minimal essential medium on the surface of the polymer disks. The cell multiplication on the surface of the specimens were measured. They reported that one heat cured resin and one pour type resin showed slight cytotoxic effect. The other polymers gave only moderate

cytotoxic effect. They reported no difference in the cytotoxicity of the polymers when manufactured by alternate processing methods.

Johnson HJ et al (1983)[24] evaluated the relative sensitivity of in-vitro biocompatibility test systems. Cellular responses of 12 standardized cell lines to 20 materials representing a range of toxicity were measured. They concluded that methods involving measurement of cellular growth (colony counts or percent of confluence) in serum-fortified media extracts of test samples were generally more sensitive and discriminating than those in which test materials were placed directly in cell cultures (measurement of zone of growth inhibition).

Stafford GD et al (1985)[25] investigated the loss of residual monomer from acrylic orthodontic resins. The results showed that high levels of residual monomer were present in orthodontic resins and there was a rapid loss of monomer in the first 24 hours of soaking of the specimens in water. This loss continued but shows that high levels of residual monomer remain.

Baker S et al (1988)[26] reported on a new assay, Gas liquid chromatography assay to estimate the release of residual monomeric methyl methacrylate from acrylic appliances in the human mouth. Using this method of estimation they concluded that the maximum amount of monomer released by an autopolymerized base plate was 29.5 micrograms in the first hour, which, while not a toxic or primary irritant dose, could possibly sensitize patients or elicit an allergic reaction. For minimization of monomer release, autopolymerized appliances should be immersed for 24 hours in water before being worn.

Hensten- Pettersen A et al (1988)[27] compared the different methods available for cytotoxicity testing and reported that toxicity testing of dental materials by means of cell culture methods has been claimed to be fairly simple to perform, reproducible, cost-effective, relevant and suitable as an alternative to animal experiments. Furthermore, these methods have been claimed to be suitable for toxicity screening of new materials, identification of cytotoxic substances and appropriate for the biological quality control of production batches. Most claims are not substantiated by

research data. They warranted further correlation studies between in-vitro tests, physical/chemical data and in-vivo studies.

Hensten-Pettersen A et al (1990)[28] evaluated the role of biomaterials as occupational hazards in dentistry and they reported that the occupational problems related to biomaterials in dentistry seem to have been fairly constant over the years, reflecting the type of materials in common use and with dermatological disorders being a tenacious companion. Neuropathological conditions in dental technicians have been associated with prolonged exposure to vapors of methyl methacrylate monomer. They stated that the more recent extensive use of volatile resin-based materials have created new problems.

Rathbun MA et al (1991)[29] reported that BIS-GMA composites, produce toxic reactions in cell culture, which could be eliminated by extraction of small amounts of leachable material from their surface in a suitable solvent. They found that these leachable material represents about one third of the weight of the organic portion of the composite.

Sheridan PJ et al(1997)[30] examined the effect of eluate from heat-activated, chemically activated and microwave-activated denture base resins on cell viability of primary cultures of human gingival fibroblasts. Eluates corresponding to 24, 48, 72, and 96 hours of resin disk immersion were prepared. They concluded that at all time periods tested, all three resins leached materials that were cytotoxic to the fibroblasts. Eluate from chemically activated resin disks was more cytotoxic than eluate from heat-activated and microwave-activated disks. In general, cytotoxicity appeared to diminish as disk immersion time was increased. The greatest cytotoxic effect on cell viability was observed with eluates recovered after 24 hours of disk immersion, and the least cytotoxic effect was observed with eluates recovered after 96 hours of immersion.

Geurtsen W et al (1998)[31] investigated the cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures. This study reported that there was no cell type identified which was consistently less

or more sensitive to the toxic effects of the tested compounds than the others. Primary human periodontal ligament and pulp fibroblasts, however, were found to be more sensitive than 3T3 and gingival fibroblasts to alterations from most tested substances.

Schmalz G et al (1999)[32] analysed the BPA content of different fissure sealant resin monomers and their release of BPA under hydrolytic conditions. They concluded that no BPA-release is expected under physiologic conditions from fissure sealants based on Bis-GMA if pure base monomers are used.

Wataha JC et al (1999)[33] tested five types of composite or compomer materials (Z-100, Tetric Ceram, Dyract AP, Solitaire, and Clearfil AP-X) and one organically modified ceramic material (Definite) after aging in artificial saliva for 0, 7, or 14 days. Cytotoxicity was assessed using direct contact with fibroblasts and measurement of succinic dehydrogenase activity after 48 hours of exposure post aging and they concluded that all of these commercially available resin-based dental materials continue to release sufficient components to cause lethal effects or alter cellular function in-vitro even after 2 weeks of aging in artificial saliva.

Kedjarune U et al (1999)[34] investigated three heat-cured and three autopolymerized acrylic denture bases with different mixing proportions and/or processing methods for the amount of residual monomer content and methyl methacrylate (MMA) released into saliva. The results showed that the amount of residual monomer was dependent not only on the type of polymerization but also on the amount of liquid in the mixture ratio and the processing method. The acrylic resin that had the lowest residual monomer also released the smallest amount of MMA but resins which have higher residual monomer may not necessarily release higher amounts of MMA.

Rose EC et al (2000)[35] investigated the cytotoxicity of orthodontic cold-cure acrylics, Orthocryl and Forestacryl, and 4 orthodontic photocure acrylics, Triad, Wil-O-Dont, Odontolux and Lux-A-Tech and compared it with 2 prosthetic acrylic materials: the cold-cure acrylic Palapress and the hot-cure acrylic Paladon. They concluded that the prosthetic cold-cure acrylic, Palapress achieved significantly better results than the orthodontic cold-cure materials, Orthocryl and Forestacryl. In the cell culture tests, all

the orthodontic materials examined were assessed as "slightly cytotoxic"; the prosthetic acrylics were graded under ISO-standard 10993-5 as "noncytotoxic".

Lee SY et al (2002)[36] investigated the influence of polymerization conditions on monomer elution and microhardness of autopolymerized polymethyl methacrylate resin and the results showed that curing temperature was the dominant factor in improving resin surface hardness, whereas curing in water was the key factor for reducing the quantity of residual monomer. The pressure factor, which was thought to be critical for managing autopolymerized resins, showed no significant influences on the properties tested.

Atkinson JC et al (2002)[37] investigated the stability of compounds of dental sealant materials in a salivary matrix. They reported that BPA was stable under all tested conditions. Samples originally containing BIS-DMA had high levels of BPA and almost no BIS-DMA after 4 months at -20 degrees C. Salivary samples incubated at 37 degrees C originally containing only BIS-DMA (200 ng/ml) demonstrated rapid decreases of BIS-DMA and increases of BPA. By 24 hours, the mean BIS-DMA concentration fell to 21.8 (25) ng/ml, while BPA increased to 100 (48) ng/ml. Only slight decreases in BIS-DMA and no BPA were present in the water samples incubated at 37 degrees C. BPA, BIS-DMA, and TEGDMA were stable if salivary samples were stored at -70 degree C. Acidification of salivary samples prevented the breakdown of BIS-DMA.

Claude G. Matasa et al (2003)[38] have done a Screening of Orthodontic Polymers for leaching. They reported that sealants are a short-term product in the oral environment, while adhesives and restorative materials remain in place for a longer period of time. Some plates and retainers may have to be repeatedly replaced (Invisalign, for example, may be replaced up to 40 times), raising the possibility of significant release of various ingredients. While these released ingredients have been reported to be toxic, mutagenic, carcinogenic and oestrogenic, there has not been a readily accessible method of assessment.

Lai YL et al (2004)[39] evaluated the cytotoxic effects of dental resin liquids on primary gingival fibroblasts and periodontal ligament cells in vitro. Cytotoxic effects of resin liquids of three in situ relining dental polymers, Alike, Kooliner, and Tokuso Rebase, and their major components, methyl methacrylate (MMA), isobutyl methacrylate (IBMA), and 1,6-hexanediol dimethacrylate (1,6-HDMA) were investigated. The results showed that all materials examined had cytotoxic effects on GF and PDL cells in dose-dependent manners. Tokuso Rebase liquid was reported to be the most cytotoxic. In conclusion, the liquid forms of dental polymers and their major monomers cause cytotoxic reactions.

Schuster S et al (2004)[40] investigated the structure of Invisalign appliances (Align Technology, Santa Clara, Calif) after intraoral exposure. They qualitatively and quantitatively characterized the substances leached from the aligners after accelerated in-vitro aging. This study demonstrated substantial morphological variations relative to the as-received specimens involving abrasion at the cusp tips, adsorption of integuments and localized calcification of the precipitated biofilm at stagnation sites. In vitro aged and retrieved appliances were found to leach no traceable amount of substances in an ethanol aging solution.

Cumhur Sipahi et al (2006)[41] studied the effect of two fibre impregnation methods on the cytotoxicity of a glass and carbon fibre-reinforced heat-polymerized acrylic resin denture base material on oral epithelial cells and fibroblasts. They reported that fibroblastic cell viability percentages of silane and monomer treated fibre reinforced groups were lower than the unreinforced group. Cell viability of monomer-treated groups displayed the lowest percentages. Elapsed incubation time decreased epithelial cell viability in silane treated groups. Fibroblastic cell viability was not influenced by elapsed time except the unreinforced group.

Julide Ozen et al (2006)[42] evaluated the in-vitro Cytotoxicity of Glass and Carbon Fiber-Reinforced Heat-Polymerized Acrylic Resin Denture Base Material and it was determined that glass and carbon fiber reinforced heat-polymerized acrylic resin was found moderately cytotoxic by decreasing the proliferation of gingival fibroblasts by approximately 20%. No difference in cytotoxicity was found between fiber-reinforced

groups and the fiber impregnation methods. The unreinforced acrylic resin was significantly less cytotoxic than the reinforced groups.

Reichl FX et al (2006)[43] investigated the cell death effects of resin-based dental material compounds and mercurials in human gingival fibroblasts. The results of this study showed that resin composite components have a lower toxicity than mercury from amalgam in HGF. HEMA, BisGMA, UDMA, and HgCl2 induced mainly necrosis, but it is rather unlikely that eluted substances (solely) can reach concentrations, which might induce necrotic cell death in the human physiological situation, indicating that other (additional) factors may be involved in the induction of tissue (pulp) inflammation effects after dental restoration

Alonso-Magdalena P (2006)[44] evaluated the estrogenic effect of bisphenol A on pancreatic beta-cell function and insulin resistance in vivo. They reported that either abnormal levels of endogenous estrogens or environmental estrogen exposure enhances the risk of developing type 2 diabetes mellitus, hypertension, and dyslipidaemia.

Jorge JH et al (2007)[45] evaluated the effects of two post-polymerization treatments and different cycles of polymerization on the cytotoxicity of two denture base resins and reported that the long cycle increased the cytotoxicity of Lucitone 550 and water-bath post-polymerisation reduced the cytotoxicity of Lucitone 550 processed by long cycle.

Gill DS et al (2007)[46] compared part-time and full-time Essix-type retainer wear regimens following fixed appliance treatment, with respect to dental alignment and occlusal changes. They reported that there was a significant reduction in overjet and overbite in only the part-time retention group during fixed appliance treatment. Between debonding and 6 months after debonding, there was no significant change in any of the intra or intergroup study cast measurements and they concluded that night-time-only Essix retainer wear may be an acceptable retention regimen following the use of fixed appliances.

Rowland H et al (2007)[47] compared the clinical effectiveness of Hawley and Vaccum Formed Retainers (VFRs) over a 6-month period of retention. They concluded that VFRs are more effective than Hawley retainers at holding the correction of the maxillary and mandibular labial segments. The median differences were 0.56 mm in the mandibular arch and 0.25 mm in the maxillary arch. They reported that this difference is clinically insignificant in the maxillary arch and significant in the mandibular arch if located to a single tooth displacement.

Vandenberg LN et al (2007)[48] studied human exposure to Bisphenol A (BPA) and they reported that the levels of BPA in human fluids are higher than the BPA concentrations reported to stimulate molecular endpoints in-vitro and appear to be within an order of magnitude of the levels needed to induce effects in animal models.

Tatiana Siqueira Goncalves et al (2008)[49] evaluated the toxic effect of different acrylic resins used in orthodontics on three established cell lines (HeLa, NIH3T3, and Hep2) and cultured under standard conditions. MTT assay was used as the cytotoxicity test. They reported that fibroblastic viability was not affected when the elution time was 24 hours, but treatments showed higher cell viability than controls when the elution time was 48 hours. When left to elute for 24 hours, both resins had cytotoxic effect on epithelial cells, but this effect was not observed when the elution time was 48 hours.

Tatiana Siqueira Goncalves et al (2008)[50] investigated the residual monomer of autopolymerized acrylic resin according to different manipulation and polishing methods. This study reported residual methyl methacrylate in high concentrations in the beginning of the testing as well as 24 hours after the test specimens had been worn. Mechanical polishing was associated with lower levels of residual monomer. The mass-mechanical group showed the lowest values.

Goncalves TS et al (2008)[51] investigated allergy to auto-polymerized acrylic resin in an orthodontic patient. They concluded that the residual monomer content was

between 0.745% and 0.78%, which did not exceed international standards for this material. Patch tests were performed with several methyl methacrylate resin samples and processed with various techniques; they showed positive reactions.

Bouskine A et al (2009)[52] explored the possible promoting effect of bisphenol A (BPA) on human testicular seminoma cells. BPA is a well-recognized estrogenic endocrine disruptor used as a monomer to manufacture poly carbonate plastic and released from resin-lined food or beverage cans or from dental sealants. They concluded that this GPCR-mediated non genomic action represents in addition to the classical ER-mediated effect a new basis for evaluating xenoestrogens such as BPA that, at low doses and with a high affinity for this GPCR, could interfere with the developmental programming of fetal germ cell proliferation and/or differentiation when they cross the placenta.

Theodore Eliades et al (2009)[53] studied the in-vitro cytotoxic and estrogenic properties of Invisalign appliances (Align Technology, Santa Clara, Calif) on human gingival fibroblasts using a modified MTT assay and concluded that there was no evidence of cytotoxicity on human gingival fibroblasts and no stimulation of proliferation of the MCF-7 cell line at any concentration, indicating no estrogenicity of aligner eluents. The use of Invisalign appliances did not seem to induce estrogenic effects under the conditions of their experiment.

Fleisch AF et al (2010)[54] assessed the presence of Bisphenol A and other related compounds in dental materials. They reported that BPA is released from dental resins through salivary enzymatic hydrolysis of BPA derivatives, and BPA is detectable in saliva for up to 3 hours after resin placement and therefore use of these materials should be minimized during pregnancy whenever possible.

Abby F. Fleisch et al (2010)[55] assessed BPA exposures from dental materials and potential health risks. Their results showed that BPA is released from dental resins through salivary enzymatic hydrolysis of BPA derivatives, and BPA is detectable in saliva for up to 3 hours after resin placement. Dental products containing the bisphenol A derivative glycidyl dimethacrylate (bis-GMA) are less likely to be

hydrolyzed to BPA and have less estrogenicity than those containing bisphenol A dimethacrylate (bis-DMA).

Ahrari F et al (2010)[56] evaluated the cytotoxic effects of a no-mix (Unite), a light-cured (Tranbond XT), and a flowable (Denfil Flow) adhesives on human oral fibroblasts. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results showed moderate cytotoxic effects of no-mix adhesive on the first day of the experiment which suggested that care should be taken to protect dentists and patients when these adhesives are being handled. Despite higher resin components, the flowable adhesive showed excellent biocompatibility.

Theodore Eliades et al (2011)[57] determined the bisphenol-A (BPA) released from a light-cured orthodontic adhesive used to bond lingual fixed retainers. They concluded that BPA was found to be released from a light-cured orthodontic adhesive bonded to a lingual fixed retainer. They reported that it may be derived from the application of this material with its surface exposed to the oral cavity, as opposed to the exposure of the marginal edges of adhesive when used as orthodontic adhesive. A temporal variation in the elution of BPA in the aging medium was noted with the highest concentration found for the 1-month immersed samples.

Ozturk F et al (2011)[58] evaluated the cytotoxicity of 3 orthodontic acrylic materials and 2 manipulation methods. The orthodontic acrylic materials Orthocryl EQ (Dentaurum, Ispringen, Germany), Orthoplast (Vertex Dental, Zeist, The Netherlands), and O-80 (Imicryl, Konya, Turkey) were prepared with 2 polymerization methods (doughing and spray on). The results indicated that the long cycle increased the cytotoxicity of the tested materials and there was no significant difference between the spray-on and doughing methods on cytotoxicity.

Sylvia Jaderberg et al (2012)[59]evaluated and compared stability after 6 months of Essix retainer use. Patients' perceptions of wearing the retainer were also evaluated. They found that the retainer was well tolerated by the patients. It was therefore

concluded that the Essix retainer is sufficient for maintaining the results after orthodontic treatment and that night time wear is adequate.

Zorana Ivankovic Buljan et al (2012)[60] determined the in vitro oxidative stress induced by conventional and self-ligating brackets made of different materials in DNA of murine fibroblast cells L929. To determine viability and changes in the number of cells before and after exposure, trypan blue dye was used. They reported that all types of orthodontic brackets, regardless of the constituent materials, are a source of oxidative stress in vitro, but the highest stress was induced in the full metal and polyurethane brackets. Conventional ceramic brackets showed the highest degree of biocompatibility compared with polymeric and metal brackets and self-ligating brackets made from combinations of these materials.

Hamada S et al (2012)[61] investigated the dermal uptake of 4, 4'-diphenylmethane diisocyanate (4, 4'-MDI) to know if it caused active sensitization. The results suggested that the volunteers were actively sensitized to 4, 4'-MDI following the dermal uptake study, as they reacted positively to 4, 4'-MDA, a marker for 4, 4'-MDI allergy. No positive reactions were seen to p-phenylenediamine(PPD) and dicyclohexylmethane 4, 4 -diisocyanate (DMDI).

Moon MK et al (2012)[62] studied the effects of Bisphenol A on mitochondrial function in the liver at doses below the adverse effect level. They found that even at these levels BPA impaired the structure of the hepatic mitochondria, although oxygen consumption rate and expression of the respiratory complex decreased only at the higher dose. So they concluded that doses of BPA below the adverse effect levels induce mitochondrial dysfunction in the liver, and this is associated with an increase in oxidative stress and inflammation.

J.C. Stockert et al (2012)[63] studied the localization of MTT formazan by direct microscopic observation of living HeLa cells and by colocalization analysis with organelle-selective fluorescent probes. MTT formazan granules did not colocalize with mitochondria as revealed by rhodamine 123 labelling or autofluorescence.

Likewise, no colocalization was observed between MTT formazan granules and lysosomes labelled by neutral red. An evaluation of the MTT reaction was performed after treatment of cells with sunflower oil emulsions to induce a massive occurrence of lipid droplets. Under this condition, lipid droplets revealed a large amount of MTT formazan deposits. Kinetic studies on the viability of MTT-treated cells showed no harmful effects at short times. Quantitative structure–activity relations (QSAR) models were used to predict and explain the localization of both the MTT tetrazolium salt and its formazan product.

Bach C et al (2013)[64] investigated the impact of temperature on the release of PET-bottle constituents into water and to assess the potential health hazard using in vitro bioassays with bacteria and human cell lines. Genotoxicity assays (Ames and micronucleus assays) and transcriptional-reporter gene assays for estrogenic and anti-androgenic activity were performed on bottled water extracts at relevant consumer exposure levels. The study reported that though phthalates nor UV stabilisers were present in the water extracts, 2,4 di-tert-butylphenol, a degradation compound of phenolic antioxidants, was detected. In addition, an intermediary monomer, bis (2-hydroxyethyl) terephthalate, was found but only in PET-bottled waters.

Luciana Borges Retamoso et al (2014)[65] assessed the in vitro cytotoxicity of acrylic resins of different colors over time.They reported that Clear, pink, blue and green self-curing acrylic resins fabricated by means of the mass manipulation technique and mechanically polished are not cytotoxic. Neither the pigment added to the self-curing acrylic resin nor the factor of time influenced the cytotoxicity of the material.

Thyagaseely Premaraj et al (2014)[14] evaluated the cellular responses of oral epithelium exposed to Invisalign plastic in vitro. The human keratinocyte N/ TERT-1 cell line was used in this study. The 3-[4, 5-dimethythiazol- 2-yl]-2, 5-diphenyl tetrazolium bromide assay and flow cytometry were used to determine cell viability and membrane integrity, respectively. Cellular adhesion and micromotion of epithelial cells were measured in real time by electrical cell-substrate impedance sensing. They reported that exposure to Invisalign plastic caused changes in viability, membrane permeability, and adhesion of epithelial cells in a saline-solution environment.

Microleakage and hapten formation secondary to compromised epithelial integrity might lead to Isocyanate allergy, which could be systemic or localized to gingiva. The results of this study suggested that saliva might offer protection.

Kotyk MW et al (2014)[66] investigated the bisphenol-A (BPA) leaching from orthodontic materials during simulated intraoral exposure. They concluded that BPA was observed to leach from two orthodontic materials. While the quantities of leached BPA were below the reference dose for daily intake, existing data of low-dose effects.

Hwa Yeon Jo et al (2015)[67] examined whether or not MTT assay can lead to incorrect information regarding alcohol-induced cytotoxicity on immortalized and primary glioblastoma cells. MTT assay was applied to assess the ethanol-induced cytotoxicity at various ethanol concentrations. Their findings demonstrated that cytotoxicity on primary cells could inaccurately be assessed when detected through MTT assay. Therefore, a careful interpretation is needed when one would analyse the cytotoxic results of MTT assay, and it is suggested that other assays must be accompanied to produce more reliable and accurate cytotoxic results on primary glioblastoma cells.

Van Tonder A et al (2015)[68] assessed the linear range and reproducibility of three commonly used cell enumeration assays; the neutral red uptake (NRU), resazurin reduction (RES) and sulforhodamine B (SRB) assays, in comparison to the MTT assay. Interference between the MTT assay and three glycolysis inhibitors, 2-deoxyglucose, 3-bromopyruvate and lonidamine, was investigated. This study demonstrated that the MTT assay was not the best assay in a number of parameters that must be considered when a cell enumeration assay is selected: the MTT assay was less accurate in detecting changes in cell number as indicated by the variation observed in the linear range, had the highest variation when the IC50 concentrations of the glycolysis inhibitors were determined, and interference between the MTT assay and all the glycolysis inhibitors tested were observed. They reported that the SRB assay performed best overall considering all of the parameters, suggesting that it is the most suitable assay for use in preclinical screening of novel therapeutic compounds with oxido-reductive potential.

Stepanenko et al (2015)[69] investigated the pitfalls of MTT assay. To test the under/overestimation of viability by the MTT assay, they compared results derived from the MTT assay with the trypan blue exclusion assay after treatment of glioblastoma U251, T98G and C6 cells with three widely used inhibitors with the known direct and side effects on energy and metabolic homeostasis - temozolomide (TMZ), a DNA-methylating agent, temsirolimus (TEM), an inhibitor of mTOR kinase, and U0126, an inhibitor of MEK1/2 kinases. Inhibitors were applied shortly as in IC50 evaluating studies or long as in studies focusing on drug resistance acquisition. Their reports showed that over/underestimation of cell viability by the MTT assay and its significance depends on a cell line, a time point of viability measurement and other experimental parameters. They provided a comprehensive survey of factors that should be accounted in the MTT assay. Their results suggested that to avoid result misinterpretation, supplementation of the tetrazolium salt-based assays with other non-metabolic assays is recommended.

Veerasathpurush Allareddy et al (2017)[70] examined adverse clinical events after the use of the Invisalign System. They reported that serious or life-threatening events could be associated with use of Invisalign systems.

Ezgi Atik et al (2017)[71] compared the effects of Essix and Hawley retainers on the acoustics of speech. They reported that the Hawley retainer affected articulatory movements in consonant–vowel combinations more prominently than the Essix retainer did. Voice onset time of the consonant [d] in the Hawley group was shorter than normal, indicating rapid articulatory movement in the alveolar region.

Al Naqbi SR et al (2018)[72] investigated the cytotoxicity and estrogenicity of Vivera retainers by assessing their biological behavioral effects as-received from the manufacturer and after retrieved from patients. They used six sets (maxillary and mandibular) of Vivera retainers, three as received and three retrieved after four weeks of use by patients. These retainers were immersed in the normal saline solution for 14 days following different modes of sterilization. The estrogenicity assays involved two cell lines, the estrogen-sensitive MCF-7 and the estrogen insensitive MDA-MB-231. They reported no significant MCF-7 proliferation induced by the three samples

compared either to the eluents from as-received retainers or to the negative control. β-estradiol induced a potent stimulation of MCF-7 cell proliferation, while no effect was observed on MDA-MB-231 cells. They concluded that the eluents of as-received and retrieved Vivera retainers did not seem to exhibit xenoestrogenic activity.

Stefano Martina et al (2019)[13] investigated the in vitro cytotoxicity of different thermoplastic materials for clear aligners on human primary gingival fibroblasts (HGFs). Four materials for clear aligners were considered in this study: Duran (Scheu-Dental GmbH, Iserlohn,Germany), Biolon (DreveDentamid GmbH, Unna, Germany), Zendura (Bay Materials LLC, Fremont, CA, USA), and SmartTrack (Align Technology, San Jose, CA, USA) and the study reported that all the materials for clear aligners presented a slight cytotoxicity. Biolon was the most cytotoxic and the thermoforming process increased the cytotoxicity of the materials.

RELEVANCE

RELEVANCE

Fixed Orthodontic treatment is always followed by a retention phase without which the results may not be stable on a long term basis. Fixed or removable retainers are used during the retention phase, among which the removable retention appliances are the most preferred. Essix and Hawley's retainers are among the most commonly used removable retention appliances. As these appliances remain in constant contact with the oral epithelium over a longer period of time, the potential effects of these appliances on the oral epithelium is very crucial.

There are no much studies on the cytotoxicity of Essix and Hawley's retainers. Studies have been done on the cytotoxicity of acrylates, but most of them reported on testing prosthodontic materials. The invisalign appliances which is produced by the same thermoplastic material as Essix retainer have been investigated on cytotoxicity in a few studies. Unlike the Invisalign appliance, the Essix retainers are worn for extended duration and hence might affect the biocompatibility of this material when used as a retainer.

METHODOLOGY

METHODOLOGY

MATERIALS USED:

1. Cold cure Acrylic polymer (DPI- RR)
2. Cold Cure Acrylic Monomer (DPI-RR)
3. Thermoplastic sheets (Duran)
4. Sterile vials (Cryohils)
5. Artificial Saliva (Meyer's Formula)
6. L929 fibroblast cell lines
7. MTT reagent (Sigma, #M2128)
8. Culture Media (GIBCO, 11965092)
9. Dimethyl Sulfoxide (Sigma, #D8418)
10. Microplates (Fisher Scientific)

EQUIPMENTS USED:

1. Pressure polymerizing Vessel (Make: Dentaurum, Germany; Model:Polyclav)
2. Biostar (Make: Scheu, Germany, Model: Biostar IV)
3. Orbital shaker
4. Microplate absorbance spectrophotometer (TECAN Infinite M Pro200)

Control Group

Description	Sample size
Cells treated with media diluted in untreated saliva	10

Test Group

Description	**Sample size**
Cells treated with media diluted in Essix retainer eluate for 1 month	10
Cells treated with media diluted in Essix retainer eluate for 2 months	10
Cells treated with media diluted in Essix retainer eluate for 4 months	10
Cells treated with media diluted in Essix retainer eluate for 6 months	10
Cells treated with media diluted in Essix retainer eluate for 8 months	10
Cells treated with media diluted in Essix retainer eluate for 1 year	10
Cells treated with media diluted in Hawley's retainer eluate for 1 month	10
Cells treated with media diluted in Hawley's retainer eluate for 2 months	10
Cells treated with media diluted in Hawley's retainer eluate for 4 months	10
Cells treated with media diluted in Hawley's retainer eluate for 6 months	10
Cells treated with media diluted in Hawley's retainer eluate for 8 months	10
Cells treated with media diluted in Hawley's retainer eluate for 1 year	10

Sample preparation

Hawley's retainer material:

Cold cure acrylic resin from DPI was used in this study. Under aseptic conditions, ten Hawley's retainer appliances were fabricated on patient casts which were polymerized according to the manufacturer's instructions. A pressure Polymeization Vessel (Dentaurum, Polyclav) was used for polymerization. All the retainers were fabricated in the Department Of Orthodontics and Dentofacial Orthopedics, St.Gregorios Dental College. Finally, these specimens were ultrasonically cleaned in distilled water for 20 minutes to kill the microorganisms that might have contaminated it during fabrication.

Essix retainer material:

Essix retainer was fabricated in Department Of Orthodontics and Dentofacial Orthopedics, St.Gregorios Dental College using a Biostar. The polyurethane sheets used for the fabrication of Essix retainer was of Duran (Scheu-Dental GmbH, Iserlohn, Germany). 10 retainer samples were fabricated in different study casts.

Control Group :

Artificial saliva was used as the control. The composition of artificial saliva which conformed to a formula given by Fusayama Meyer is given below.

Table1- Composition of Artificial Saliva

Composition	**Amount (gm/lit)**
Sodium Chloride (NaCl)	0.4
Potassium Chloride (KCl)	0.4
Calcium Chloride ($CaCl_2.2H_2O$)	0.8
Sodium di hydrogen phosphate ($NaH_2PO_4.2H_2O$)	0.78
Sodium Sulfide ($NaS.9H_2O$)	0.005
Urea	1

Elute preparation

The Hawley's retainer and Essix retainer material were powdered using a file. The Essix eluate was prepared by soaking 0.1 g of powdered material into 1 ml of artificial saliva in sterile vials .The samples were tested at different time intervals: 1 month, 2 months, 3 months, 4 months, 6 months, 8 months and 1 year. Similarly the cold cure acrylic eluate was prepared by soaking 0.1 g of powdered material into 1 ml of artificial saliva in sterile vials and tested at time intervals of 1 month, 2 months, 3 months, 4 months, 6 months, 8 months and 1 year.

Cell viability assessment

The evaluation of cytotoxicity was done in the Cancer Research Laboratory at Rajiv Gandhi Center for Biotechnology, Trivandrum. L929 mouse fibroblast cell lines were used for cytotoxicity testing. Cell viability was evaluated by the MTT assay, which is based on the ability of the mitochondrial enzyme succinate dehydrogenase to convert the yellow water-soluble tetrazolium salt (MTT) into formazan crystals in metabolically active cells. This water-insoluble, dark blue product is stored in the cytoplasm of cells, and is soluble afterwards, generating a blue color. The color intensity is directly proportional to the amount of viable cells.

MTT Assay Protocol

1. L929 cells were trypsinized (Himedia, TCL-070) and seeded at a density of 3000 cells per well in 96 well plates (Fisher Scientific).
2. For each condition to be tested, the cells were seeded in triplicates.
3. The cells were incubated overnight in 200 µl of media (GIBCO, 11965092) with 10% Fetal Bovine Serum (GIBCO, 10082147) and 1% antibiotic Penstrep (GIBCO, 15140122).
4. After incubation, the culture supernatant was removed and 200 µl of media containing 1:4 dilution of all the test samples were added and incubated for 24hours at 37°C in 5% CO_2 incubator.
5. After 24 hours the treatment cells were incubated with MTT (20 µl; 5 mg/ml) (Sigma, #M2128) at 37°C for 4 hours.

6. The supernatant was removed and 100 µl/well of Dimethyl Sulfoxide (Sigma, #D8418) was added. It acted both as a solubilizer of the purple crystals and as a cell lysis buffer, breaking down the cells and freeing the purple crystals.
7. The microplate was then kept on an orbital shaker for 20 minutes to dissolve the formed formazan crystals.
8. The concentration of crystals was measured by Optical Density evaluation in a microplate absorbance spectrophotometer (TECAN Infinite M Pro200) using a wavelength of 490 nm, while subtracting the Optical Density readings of a reference wavelength of 655 nm to eliminate background.
9. Cell viability of a given sample was calculated as the percentage of the relation of Optical Density of treated experimental cells to that of the cells treated with the control group. An average value of the triplicate wells were obtained for each condition.

The optical density of the cells cultured in the DMEM medium and treated with the artificial saliva served as a control for 100% cell viability and as a reference for the determination of the level of cytotoxicity in the assay. The Optical Density was measured at 490 and 655 nm in order to get a more exact measurement by correcting for background noise.

Cell proliferation was calculated using the formula below.

Percentage of Cell proliferation = OD of the Test Group ÷ OD of the control X 100

Cell viability was then scored according to the classification of Ahrari et al.

- Cell viability more than 90% : No cytotoxicity
- Cell viability of 60-90% : Slight cytotoxicity
- Cell viability of 30%–59% : Moderate cytotoxicity
- Cell viability of less than 30% : Severe cytotoxicity

Figure 1: The Cold cure acrylic material that was used for the fabrication of Hawley's retainer.

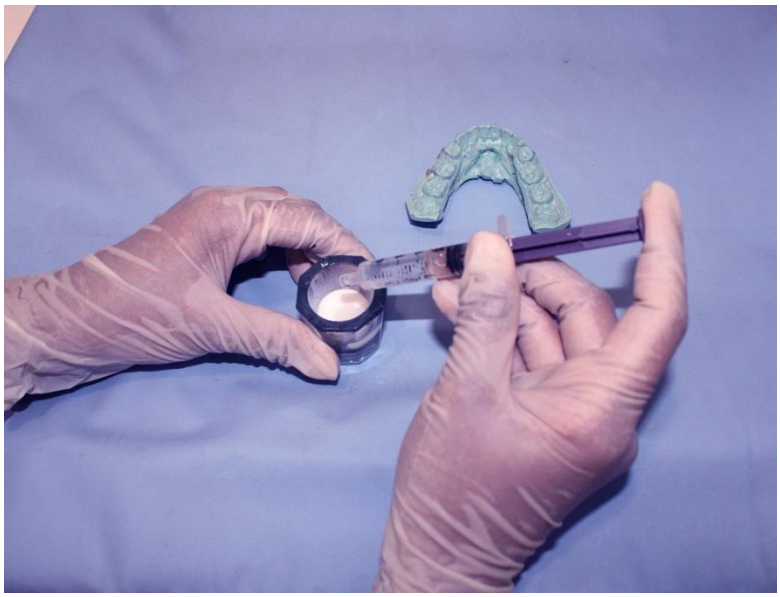


Figure 2: Mixing of cold cure acrylic material for the fabrication of Hawley's retainer.

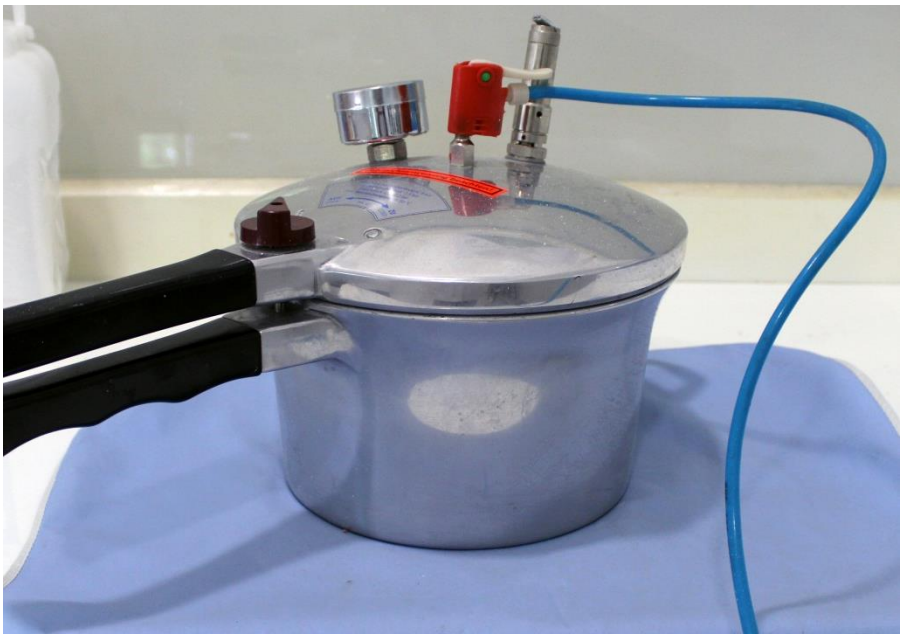


Figure 3: Pressure polymerization Vessel (polyclav-Dentaurum) used for the acrylic resin polymerization.

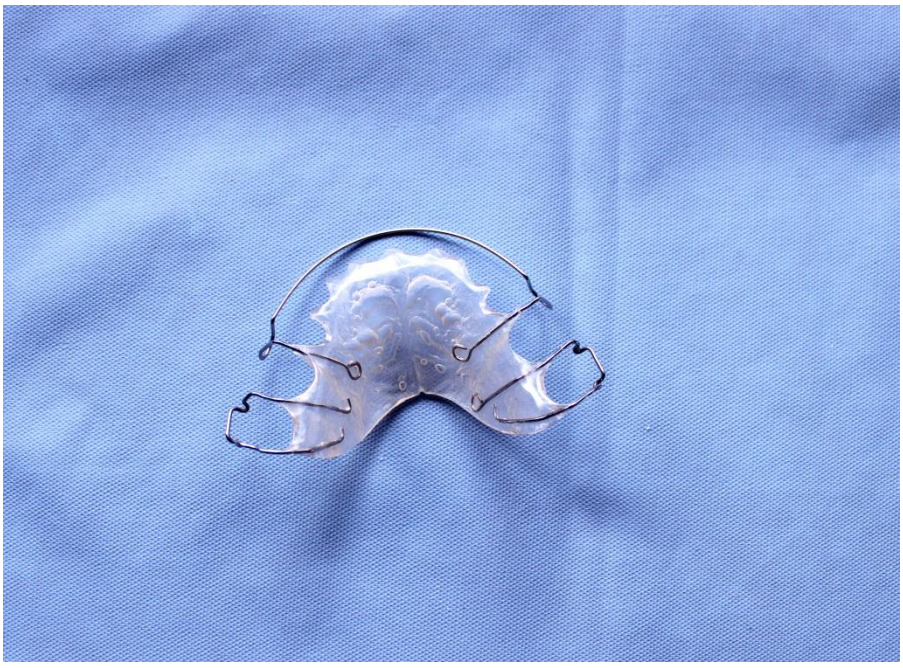


Figure 4: Hawley's retainer sample fabricated using Cold cure Acrylic material.

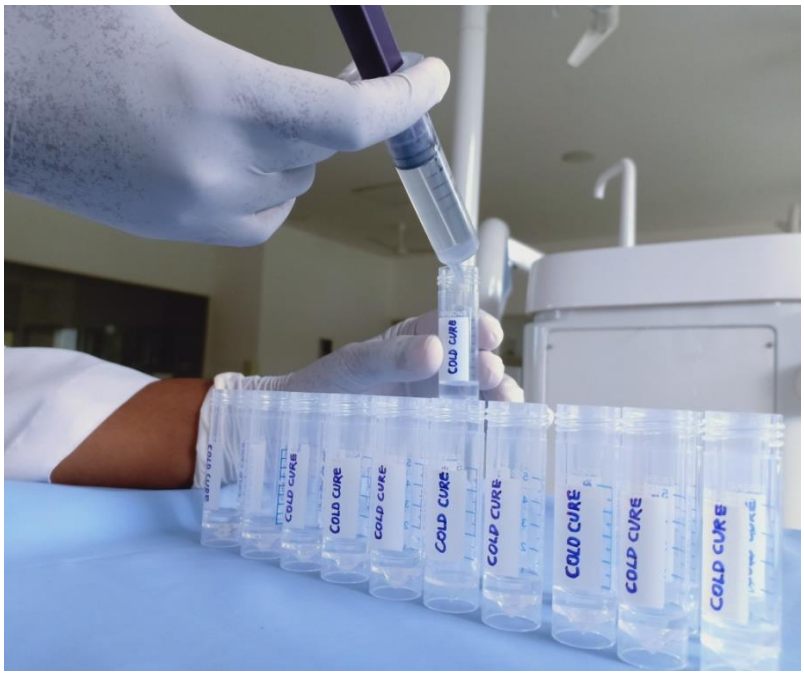


Figure 5: The sterile vials (cryohils) being filled with 1 ml of artificial saliva.

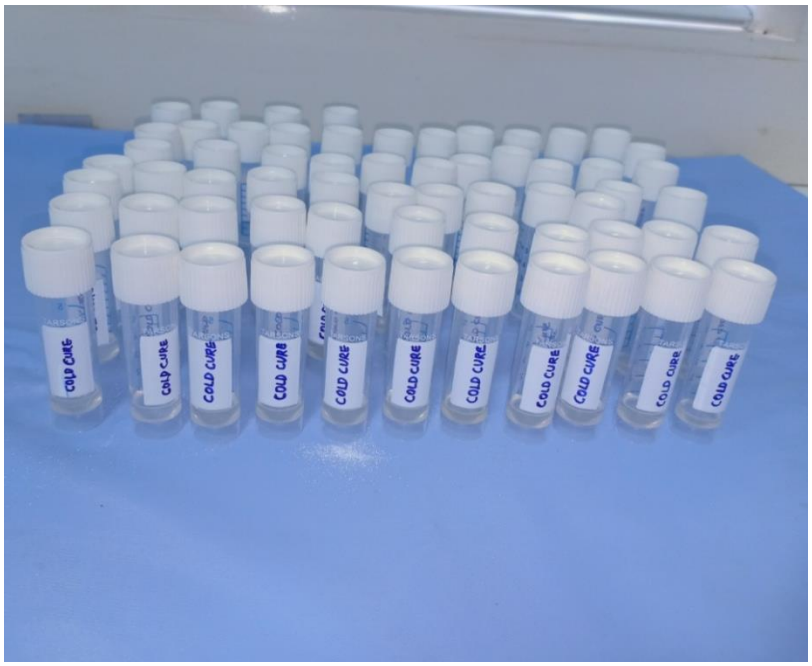


Figure 6 : Sterile vials (Cryohils) with particulated cold cure material soaked in 1 ml artificial saliva.

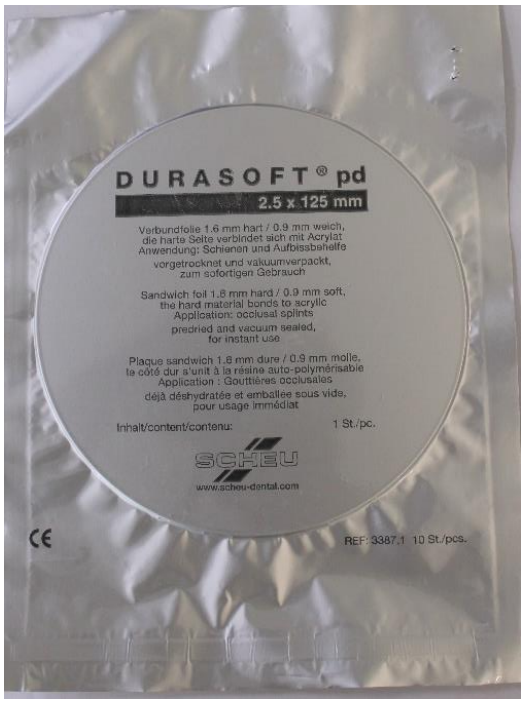


Figure 7 : The polyurethane sheet (DURAN) used in the fabrication of Essix retainer.

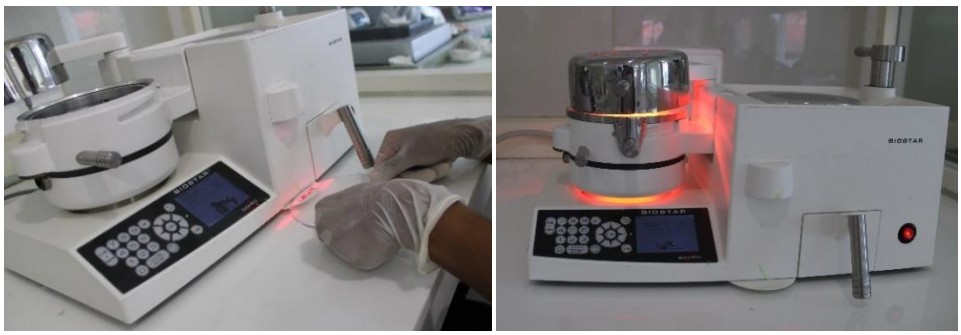


Figure 8, 9: The Biostar (Make:Scheu, Germany, Model: Biostar IV), the equipment which was used in the fabrication of Essix Retainer.

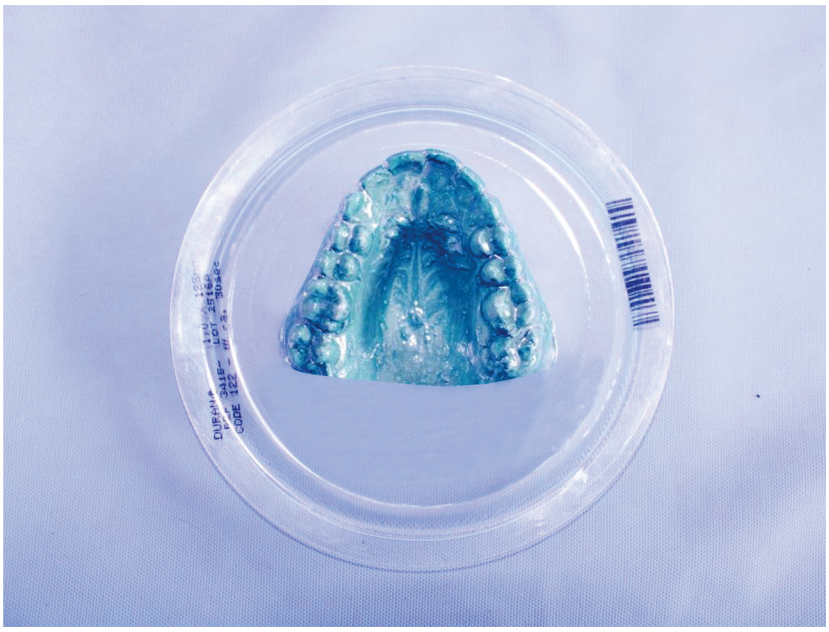
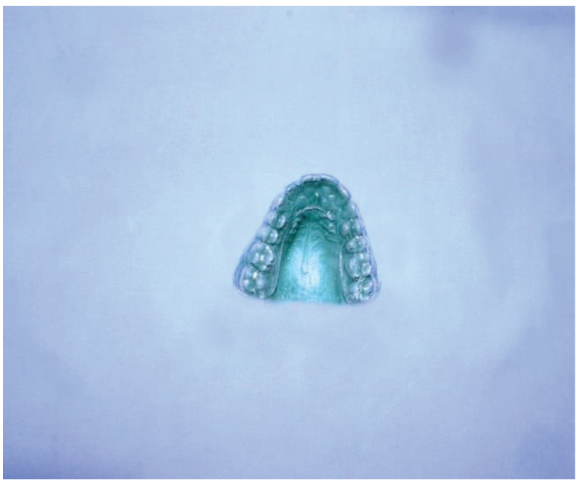


Figure 10: The untrimmed Essix retainer.



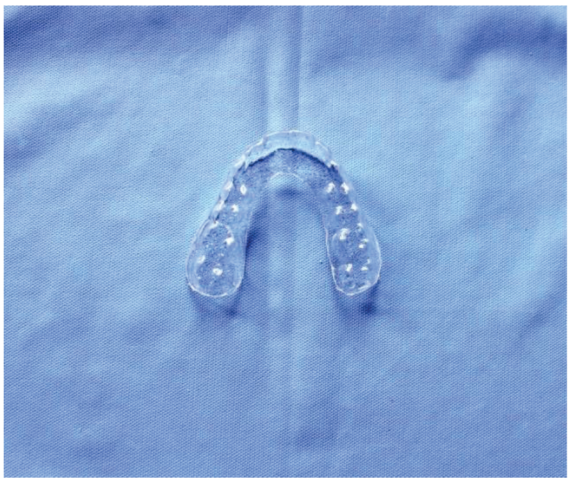


Figure 11, 12: The trimmed Essix retainer.

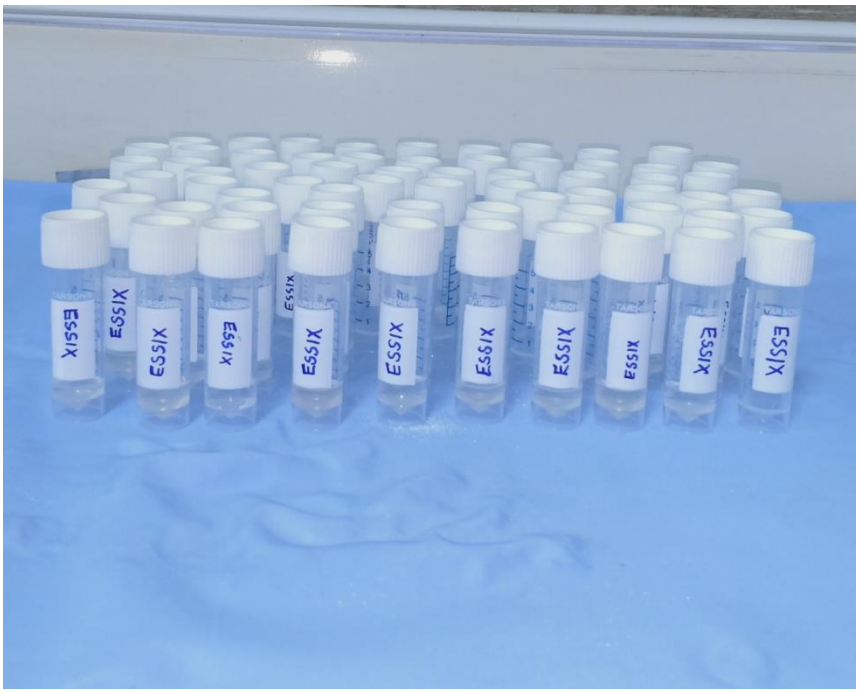


Figure 13: The sterile vials with particulated Essix material soaked in 1 ml artificial saliva.

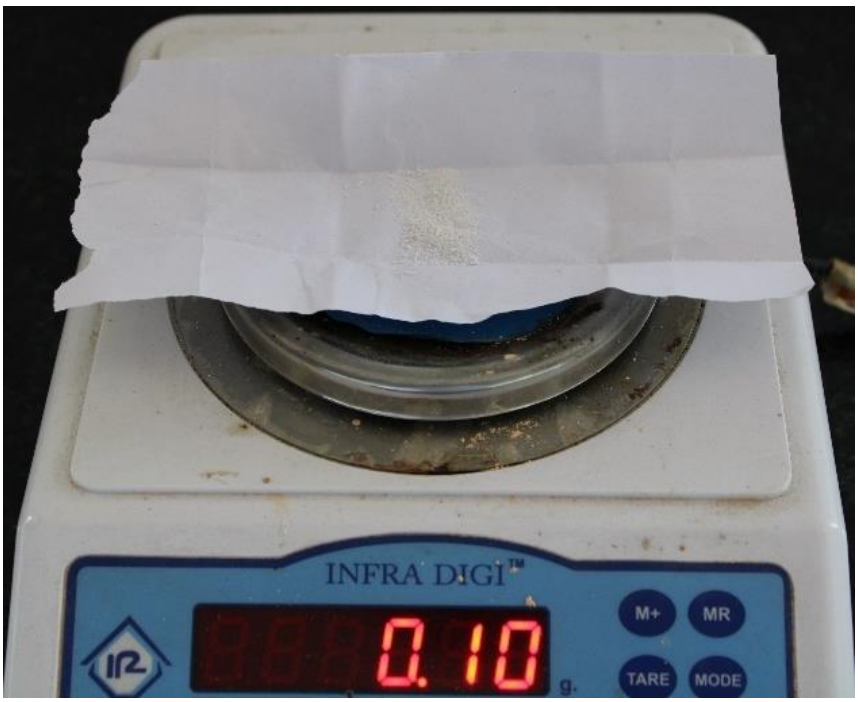


Figure 14: Measurement of the particulated samples.

Figure 15: The microplates (Thermo Fisher Scientific-Nunclon 96 Flat Bottom Transparent Polystyrene) used in MTT assay.

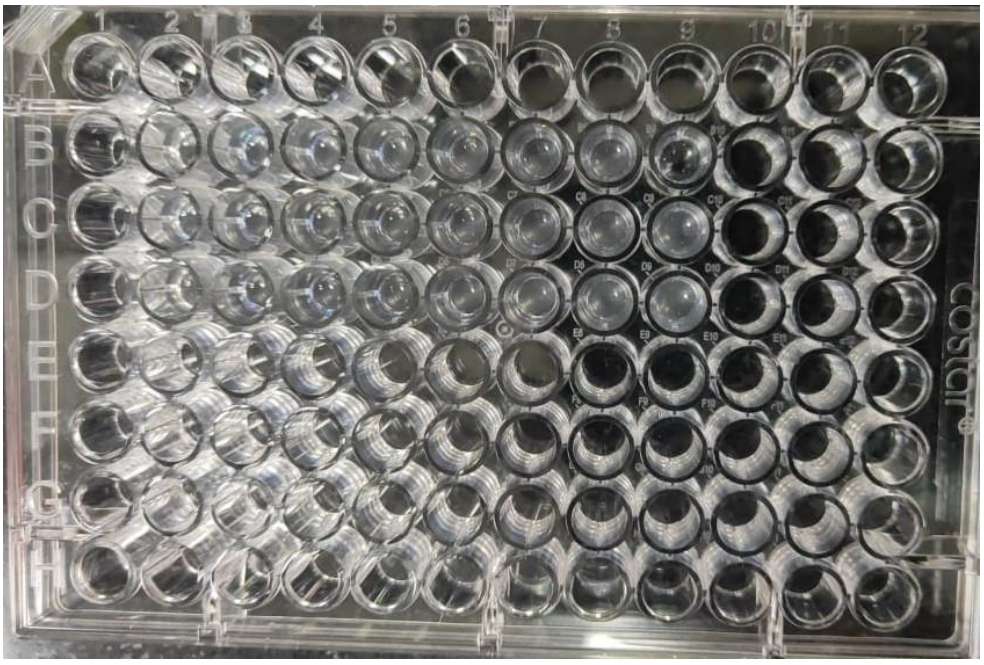


Figure 16: The microplate wells ready to be seeded with L929 fibroblast cells.

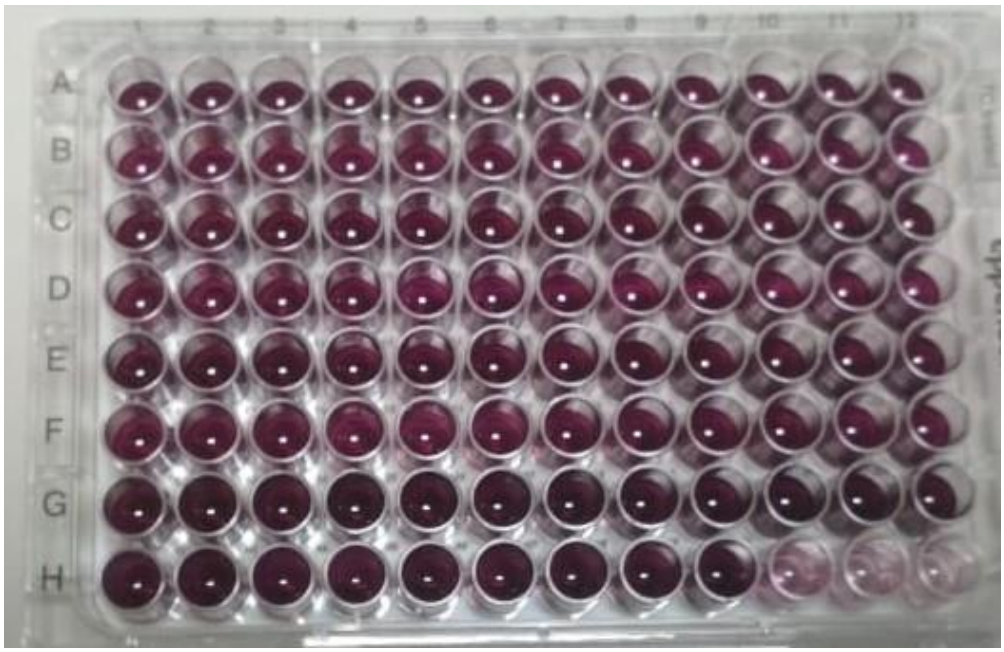


Figure 17: The microplate wells with formation of purple coloured formazan after the cells are treated with the samples and addition of MTT reagent.

Figure 18: The microplate reader (Tecan Infinite M200) used to measure the optical density.

Microscopic images of the Samples after MTT assay

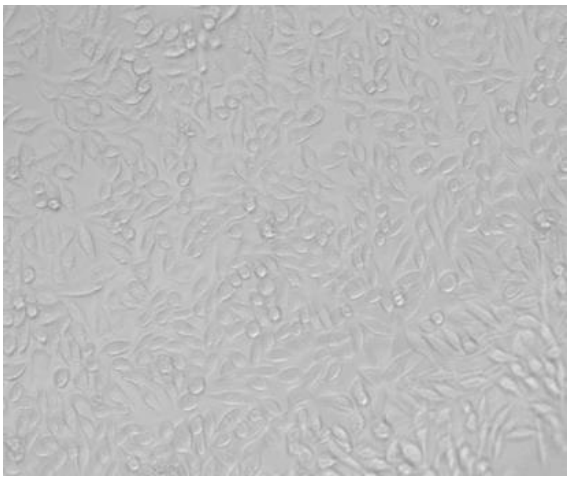


Figure 19: L929 fibroblast cell morphology after exposure to control group at 1 month.

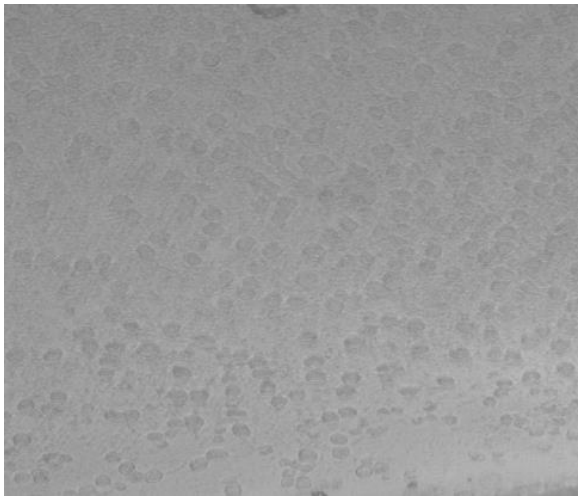


Figure 20: L929 fibroblast cell morphology after exposure to Cold cure material at 1 month.

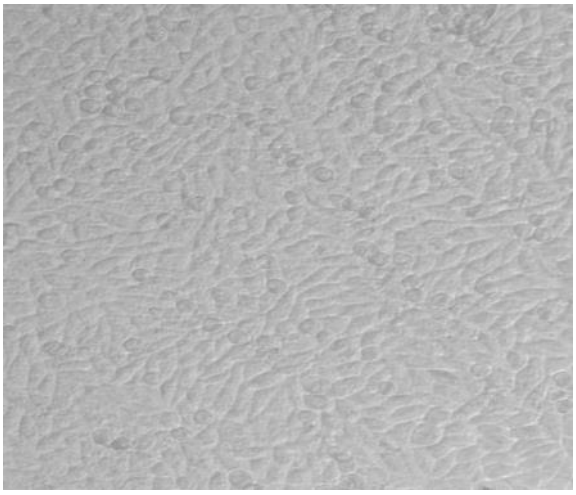


Figure 21: L929 fibroblast cell morphology after exposure to Essix retainer material at 1 month.

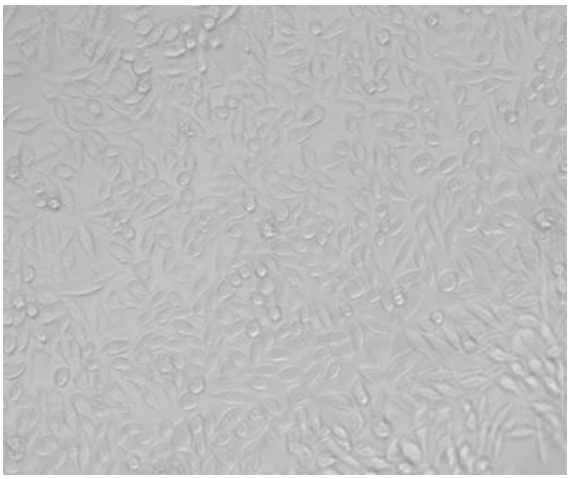


Figure 22: L929 fibroblast cell morphology after exposure to control group at 2 months.

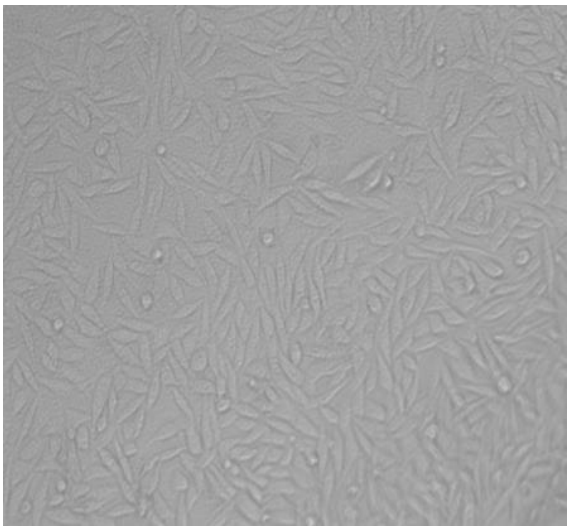


Figure 23: L929 fibroblast cell morphology after exposure to cold cure acrylic material at 2 months.

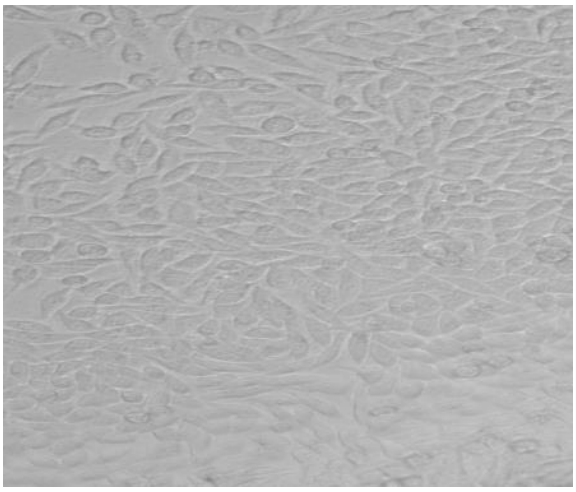


Figure 24: L929 fibroblast cell morphology after exposure to Essix retainer material at 2 months.

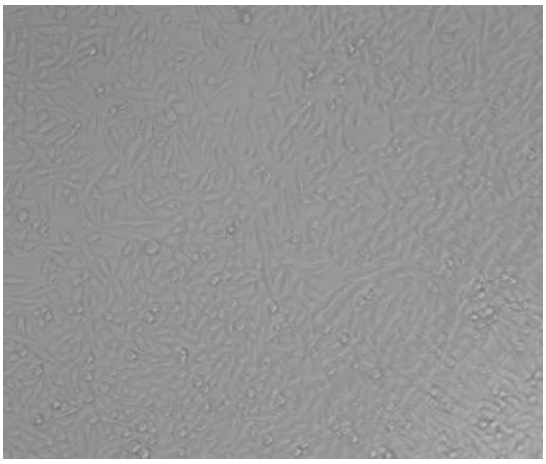


Figure 25: L929 fibroblast cell morphology after exposure to control group at 4 months.

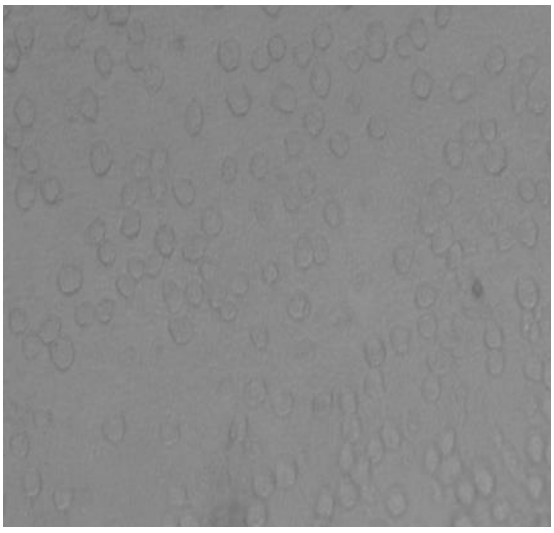


Figure 26: L929 fibroblast cell morphology after exposure to cold cure acrylic material at 4 months.

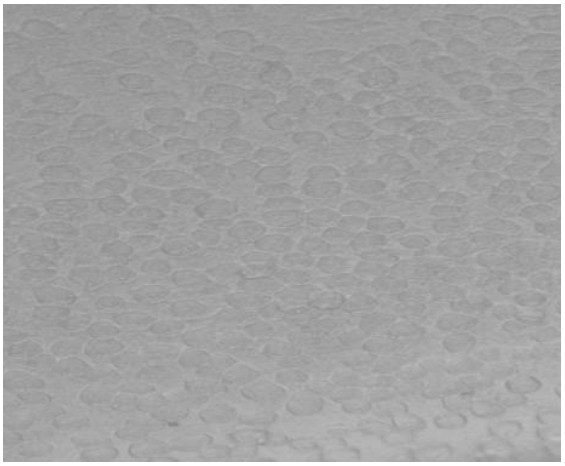


Figure 27: L929 fibroblast cell morphology after exposure to Essix retainer material at 4 months.

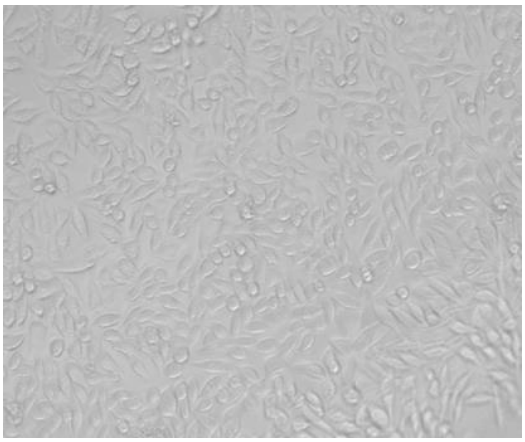


Figure 28: L929 fibroblast cell morphology after exposure to control group at 6 months.

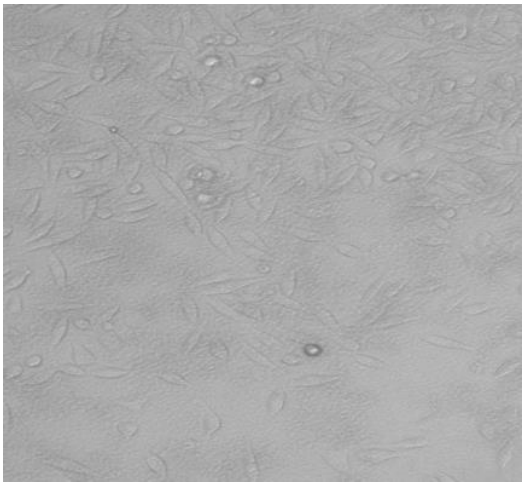


Figure 29: L929 fibroblast cell morphology after exposure to cold cure acrylic material at 6 months.

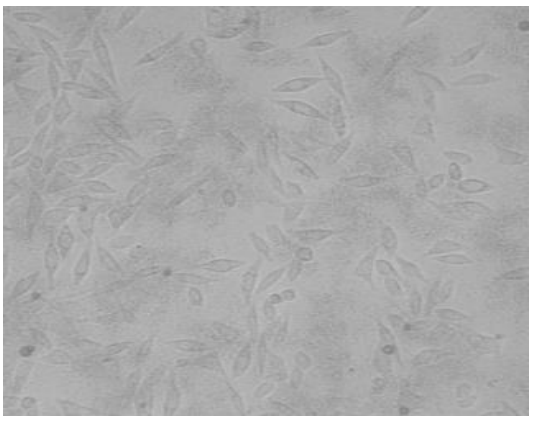


Figure 30: L929 fibroblast cell morphology after exposure to Essix retainer material at 6 months.

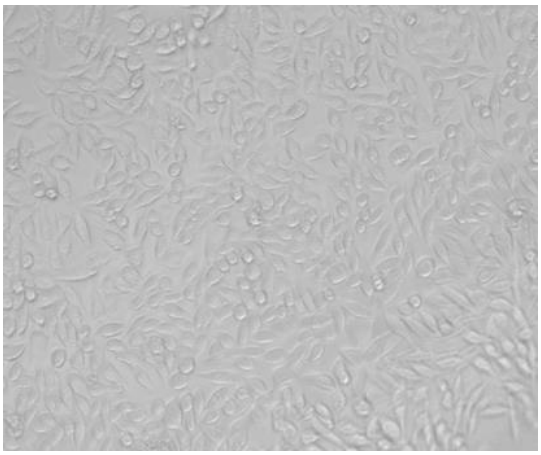


Figure 31: L929 fibroblast cell morphology after exposure to control group at 8 months.

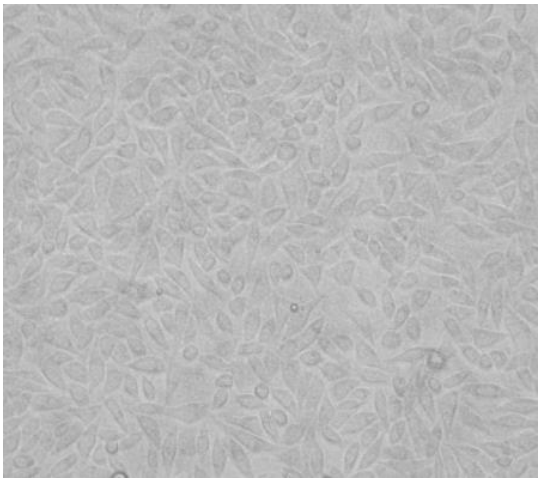


Figure 32: L929 fibroblast cell morphology after exposure to cold cure acrylic material at 8 months.

Figure 33: L929 fibroblast cell morphology after exposure to Essix retainer material at 8 months.

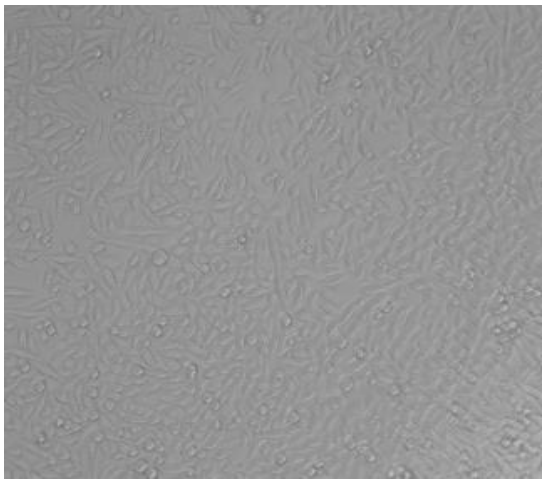


Figure 34: L929 fibroblast cell morphology after exposure to control group at 1 year.

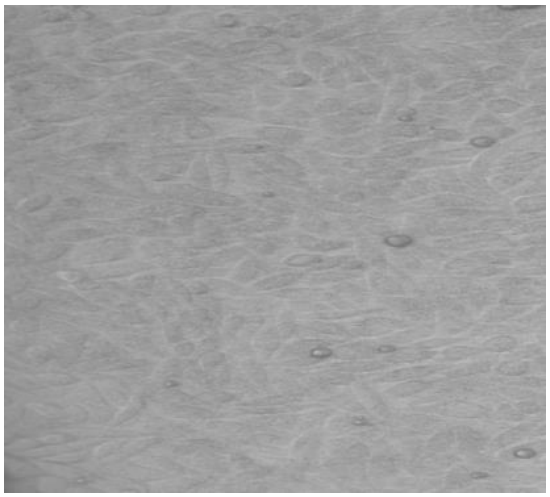


Figure 35: L929 fibroblast cell morphology after exposure to cold cure acrylic material at 1 year.

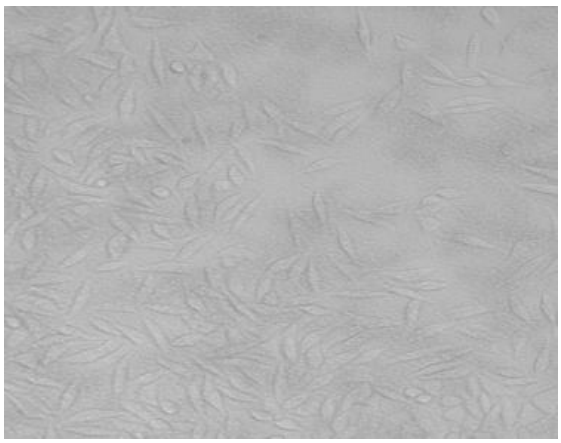


Figure 36: L929 fibroblast cell morphology after exposure to Essix retainer material at 1 year.

RESULTS

RESULTS

Table 2- Cell Viability Mean & SD (Cold Cure &Essix group)

	COLD CURE		ESSIX	
	MEAN	**SD**	**MEAN**	**SD**
1 MONTH	435.466	172.46	479.228	137.866
2 MONTHS	379.362	56.488	375.362	47.442
4 MONTHS	332.418	33.729	373.836	80.243
6 MONTHS	443.116	100.853	460.048	102.693
8 MONTHS	316.338	39.621	116.493	6.965
1 YEAR	276.973	48.87	97.002	10.330

Table 2: The mean cell viability of 10 samples of cold cure and Essix group at 6 time intervals.

Table 3- Optical Density Mean & SD (Cold Cure, Essix & Control group)

	COLD CURE		ESSIX		CONTROL	
	MEAN	**SD**	**MEAN**	**SD**	**MEAN**	**SD**
1 MONTH	1.2728	0.3384	1.1767	0.0518	0.266	0.014
2 MONTHS	1.190	0.174	1.1833	0.1236	0.309	0.016
4 MONTHS	1.2464	0.1204	1.4309	0.3310	0.379	0.014
6 MONTHS	1.2593	0.2358	1.2756	0.2950	0.283	0.012
8 MONTHS	1.3417	0.1734	0.4880	0.0312	0.420	0.008
1 YEAR	1.3269	0.2251	0.4734	0.0456	0.198	0.058

Table 3: The mean optical density of 10 samples of cold cure acrylic, Essix retainer and Control group at 6 time intervals.

Statistical Analysis

Data was analyzed using the statistical package SPSS 22.0 (SPSS Inc., Chicago, IL) and level of significance was set at $p<0.05$. Descriptive statistics was performed to assess the mean and standard deviation of the respective groups. Normality of the data was assessed using Shapiro Wilkinson test. Inferential statistics to find out the difference between the groups was done using one- way ANOVA test followed by Tukey's Post hoc analysis to find out the difference between any two groups. Independent t test was done to find out the significant difference between the groups.

Table 4- Comparison of Cell Viability between the Groups

	COLD CURE		ESSIX		P value	T value
	MEAN	**SD**	**MEAN**	**SD**		
1 MONTH	435.466	172.46	479.228	137.866	0.538	0.627
2 MONTHS	379.362	56.488	375.362	47.442	0.865	0.171
4 MONTHS	332.418	33.729	373.836	80.243	0.147	1.514
6 MONTHS	443.116	100.853	460.048	102.693	0.711	0.376
8 MONTHS	316.338	39.621	116.493	6.965	**0.001***	16.028
1 YEAR	276.973	48.87	97.002	10.330	**0.001***	11.548

Table 4: Comparison of cell viability between the cold cure and Essix groups using Independent t test which indicates significant difference at 8 months & 12 months.

***P<0.05 is statistically significant (Independent t test)**

Table 5- Within the Group Comparison

	COLD CURE		P value
	MEAN	**SD**	
1 MONTH	435.466	172.46	**0.003***
2 MONTHS	379.362	56.488	
4 MONTHS	332.418	33.729	
6 MONTHS	443.116	100.853	
8 MONTHS	316.338	39.621	
1 YEAR	276.973	48.87	

Table 5: Comparison of cell viability values within the cold cure acrylic group. The P value was found to be 0.003.

Table 6- The Results of the One-Way ANOVA are given below.

	Sum of squares	**Df**	**Mean Squares**	**F**	**Sig**
Between Groups	224437.5216	5	44887.5043	5.5879	**0.0003***
Within Groups	433777.7600	54	8032.9215		
Total	658215.2816	59			

Table 6: The results of the One-way ANOVA of cell viability values within the cold cure acrylic group.

Table 7- Post Hoc Tukey's HSD Test

Group(I)	Group(J)	Mean difference (I-J))	95% of confidence interval		P VALUE
			Lower	upper	
1 month	2 months	-56.1040	-174.5276 to	62.3196	0.7270
	4 months	-103.0480	-221.4716 to	15.3756	0.1223
	6 months	7.6500	-110.7736 to	126.0736	1.0000
	8 months	-119.1280	-237.5516 to	-0.7044	0.0478*
	1 year	-158.4830	-276.9066 to	-40.0594	0.0029*
2 months	4 months	-46.9440	-165.3676 to	71.4796	0.8484
	6 months	63.7540	-54.6696 to	182.1776	0.6082
	8 months	-63.0240	-181.4476 to	55.3996	0.6199
	1 year	-102.3790	-220.8026 to	16.0446	0.1268
4 months	6 months	110.6980	-7.7256 to	229.1216	**0.0796**
	8 months	-16.0800	-134.5036 to	102.3436	0.9986
	1 year	-55.4350	-173.8586 to	62.9886	0.7368
6 months	8 months	-126.7780	-245.2016 to	-8.3544	**0.0292***
	1 year	-166.1330	-284.5566 to	-47.7094	**0.0016***
8 months	1 year	-39.3550	-157.7786 to	79.0686	0.9217

Table 7: The Post Hoc Tukey's Test of the cell viability values within the cold cure acrylic group.

***P<0.05 is statistically significant**

Post Hoc Analysis of the cold cure acrylic group shows significant difference between 1 month vs 8 months ,1 month vs 1 year, 6 months vs 8 months, 6 months vs 1 year

Table 8-Within the Group Comparison

	ESSIX		P value
	MEAN	**SD**	
1 MONTH	479.228	137.866	0.001*
2 MONTHS	375.362	47.442	
4 MONTHS	373.836	80.243	
6 MONTHS	460.048	102.693	
8 MONTHS	116.493	6.965	
1 YEAR	97.002	10.330	

Table 8: Comparison of cell viability values within Essix retainer group at different time intervals.

Table 9- The results of the one-way ANOVA are given below.

	Sum of squares	**Df**	**Mean Squares**	**F**	**sig**
Between Groups	1420202.3638	5	284040.4728	44.3775	0.001*
Within Groups	345629.7340	54	6400.5506		
Total	1765832.0979	59			

Table 9: The one-way ANOVA results of the cell viability values within the Essix group.

Table 10- Post Hoc Tukey's HSD Test

Group(I)	Group(J)	Mean difference (I-J)	95% of confidence interval		P Value
			Lower	upper	
1 month	2 months	-103.8660	-209.5746 to	1.8426	0.0568
	4 months	-105.3660	-211.0746 to	0.3426	0.0512
	6 months	-19.1800	-124.8886 to	86.5286	0.9944
	8 months	-362.7300	-468.4386 to	-257.0214	**0.0000***
	1 year	-382.2260	-487.9346 to	-276.5174	0.0000*
2 months	4 months	-1.5000	-107.2086 to	104.2086	1.0153
	6 months	84.6860	-21.0226 to	190.3946	0.1863
	8 months	-258.8640	-364.5726 to	-153.1554	0.0000*
	1 year	-278.3600	-384.0686 to	-172.6514	0.0000*
4 months	6 months	86.1860	-19.5226 to	191.8946	0.1715
	8 months	-257.3640	-363.0726 to	-151.6554	0.0000*
	1 year	-276.8600	-382.5686 to	-171.1514	0.0000*
6 months	8 months	-343.5500	-449.2586 to	-237.8414	0.0000*
	1 year	-363.0460	-468.7546 to	-257.3374	0.0000*
8 months	1 year	-19.4960	-125.2046 to	86.2126	0.9940

Table 10: Post Hoc Tukey's HSD test of the cell viability values within the Essix group

***P<0.05 is statistically significant.**

Post hoc analysis of the Essix retainer group shows significant difference between 1 month vs 8 months ,1 month vs 1 year, 2 months vs 8 months , 2 months vs 1 year, 4 months vs 8 months,4 months vs 1 year, 6 months vs 8 months, 6 months vs 1 year

Table 11- Comparison of Optical Density (Cold Cure Vs Control)

	COLD CURE		CONTROL		P value	T value
	MEAN	**SD**	**MEAN**	**SD**		
1 MONTH	1.2728	0.3384	0.266	0.014	**0.001***	9.403
2 MONTHS	1.190	0.174	0.309	0.016	**0.001***	15.944
4 MONTHS	1.2464	0.1204	0.379	0.014	**0.001***	22.693
6 MONTHS	1.2593	0.2358	0.283	0.012	**0.001***	13.116
8 MONTHS	1.3417	0.1734	0.420	0.008	**0.001***	16.817
1 YEAR	1.3269	0.2251	0.198	0.058	**0.001***	15.351

Table 11: Comparison of optical density value between cold cure acrylic and control groups. It showed significant difference at all time intervals.

***P<0.05 is statistically significant (Independent t test)**

Table 12-Comparison of Optical Density (Essix Vs Control)

	ESSIX		CONTROL		P value	T value
	MEAN	**SD**	**MEAN**	**SD**		
1 MONTH	1.1767	0.0518	0.266	0.014	**0.001***	56.34
2 MONTHS	1.1833	0.1236	0.309	0.016	**0.001***	22.28
4 MONTHS	1.4309	0.3310	0.379	0.014	**0.001***	10.03
6 MONTHS	1.2756	0.2950	0.283	0.012	**0.001***	10.62
8 MONTHS	0.4880	0.0312	0.420	0.008	**0.001***	6.92
1 YEAR	0.4734	0.0456	0.198	0.058	**0.001***	13.58

Table 12: Comparison of optical density values between Essix and Control group. It showed significant difference at all time intervals.

***P<0.05 is statistically significant (Independent t test)**

Table 13- Within Group Comparison

	COLD CURE		P value
	MEAN	**SD**	
1 MONTH	1.2728	0.3384	0.659
2 MONTHS	1.190	0.174	
4 MONTHS	1.2464	0.1204	
6 MONTHS	1.2593	0.2358	
8 MONTHS	1.3417	0.1734	
1 YEAR	1.3269	0.2251	

Table 13: Comparison of optical density values within the cold cure acrylic group at different time intervals.

Table 14- The results of the one-way ANOVA are given below.

	Sum of squares	**Df**	**Mean Squares**	**F**	**sig**
Between Groups	0.1541	5	0.0308	0.6548	0.6591
Within Groups	2.5416	54	0.0471		
Total	2.6957	59			

Table 14: Results of the One-way ANOVA of optical density values within the cold cure acrylic group.

Table 15-Post Hoc Tukey's HSD Test

Group(I)	Group(J)	Mean difference (I-J)	95% of confidence interval		P VALUE
			Lower	upper	
1 month	2 months	-0.0828	-0.3695 to	0.2039	0.9557
	4 months	-0.0264	-0.3131 to	0.2603	0.9998
	6 months	-0.0135	-0.3002 to	0.2732	1.0000
	8 months	0.0689	-0.2178 to	0.3556	**0.9799**
	1 year	0.0541	-0.2326 to	0.3408	0.9933
2 months	4 months	0.0564	-0.2303 to	0.3431	0.9919
	6 months	0.0693	-0.2174 to	0.3560	0.9794
	8 months	0.1517	-0.1350 to	0.4384	0.6255
	1 year	0.1369	-0.1498 to	0.4236	0.7203
4 months	6 months	0.0129	-0.2738 to	0.2996	1.0000
	8 months	0.0953	-0.1914 to	0.3820	0.9216
	1 year	0.0805	-0.2062 to	0.3672	0.9607
6 months	8 months	0.0824	-0.2043 to	0.3691	0.9566
	1 year	0.0676	-0.2191 to	0.3543	0.9815
8 months	1 year	-0.0148	-0.3015 to	0.2719	1.0000

Table 15: Post Hoc Analysis of optical density values within the cold cure acrylic group.

***P<0.05 is statistically significant**

Post hoc analysis shows no significant difference between any pair comparison.

Table 16- Within Group Comparison of Optical Density (Control)

	CONTROL		P value
	MEAN	**SD**	
1 MONTH	0.266	0.014	**0.001***
2 MONTHS	0.309	0.016	
4 MONTHS	0.379	0.014	
6 MONTHS	0.283	0.012	
8 MONTHS	0.420	0.008	
1 YEAR	0.198	0.058	

Table 16: Comparison of optical density within the control group at different time intervals.

Table 17- The results of the one-way ANOVA are given below.

	Sum of squares	df	Mean Squares	F	Sig
Between Groups	0.3207	5	0.0641	91.1853	**0.001***
Within Groups	0.0380	54	0.0007		
Total	0.3586	59			

Table 17: The results of one way ANOVA of optical density values within the control group.

Table 18- Post Hoc Tukey's HSD Test

Group(I)	Group(J)	Mean difference (I-J))	95% of confidence interval		P VALUE
			Lower	upper	
1 month	2 months	0.0430	0.0080 to	0.0780	0.0080
	4 months	0.1130	0.0780 to	0.1480	0.0000*
	6 months	0.0170	-0.0180 to	0.0520	0.7068
	8 months	0.1540	0.1190 to	0.1890	**0.0000***
	1 year	-0.0680	-0.1030 to	-0.0330	0.0000*
2 months	4 months	0.0700	0.0350 to	0.1050	0.0000*
	6 months	-0.0260	-0.0610 to	0.0090	0.2583
	8 months	0.1110	0.0760 to	0.1460	0.0000*
	1 year	-0.1110	-0.1460 to	- 0.0760	0.0000*
4 months	6 months	-0.0960	-0.1310 to	-0.0610	0.0000*
	8 months	0.0410	0.0060 to	0.0760	0.0130
	1 year	-0.1810	-0.2160 to	-0.1460	0.0000*
6 months	8 months	0.1370	0.1020 to	0.1720	0.0000*
	1 year	-0.0850	-0.1200 to	-0.0500	0.0000*
8 months	1 year	-0.2220	-0.2570 to	-0.1870	0.0000*

Table 18: Post Hoc Analysis of optical density values within the control group.

***P<0.05 is statistically significant**

Post hoc analysis of control group shows significant difference between 1 month vs 4 months, 1 month vs 8 months, 1 month vs 1 year, 2 months vs 4 months, 2 months vs 8 months, 2 months vs.1 year, 4 months vs 6 months, 4 months vs 1 year, 6 months vs 8 months, 6 months vs 1 year, 8 months vs 1 year

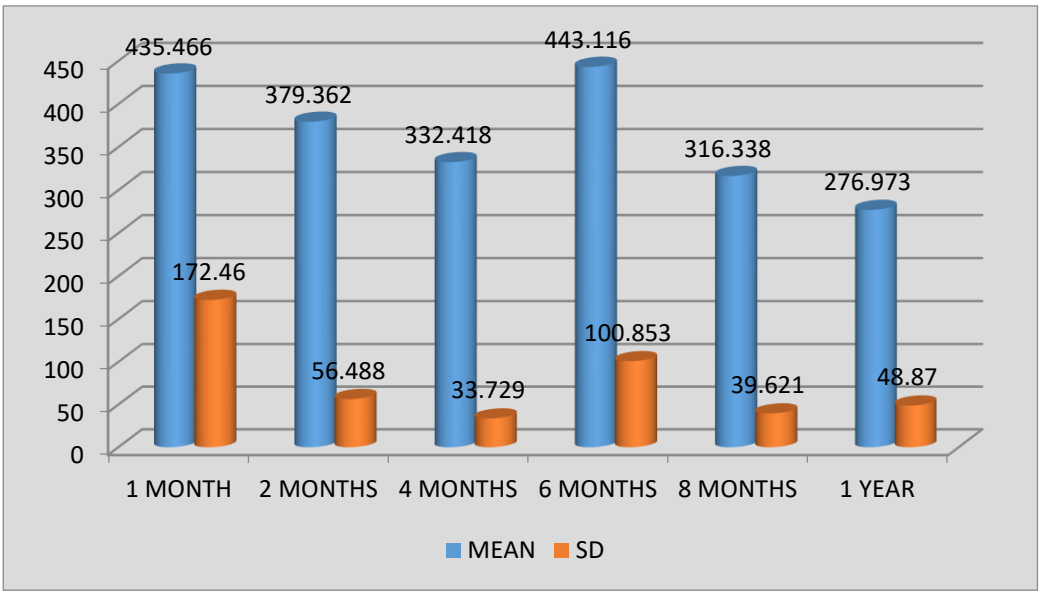
Interpretation of The statistical Analysis

Statistical analysis with independent t test to compare the cell viability among different groups at different time intervals showed a significant (p value<0.05) difference in the 8 months and 1 year samples of Essix retainer. Within the cold cure acrylic group itself, cell viability values during different time interval showed statistically significant difference. One Way ANOVA and Post Hoc Tukey's HSD test revealed significant difference between 1month vs 8 months, 1 month vs 1 year, 6 months vs 8 months and 6 months vs 1 year samples. Comparison of the cell viability values within the Essix retainer group showed statistically significant difference. one way ANOVA and Post Hoc Tukey's HSD test was done and the results showed significant difference between 1 month vs 8 months,1 month vs 1 year, 2 months vs 8 months, 2 months vs 1 year, 4 months vs 8 months, 4 months vs 1 year, 6 months vs 8 months and 6 months vs 1 year samples.

Comparison of optical density values between cold cure acrylic and control groups showed significant difference at all time intervals. Optical density comparison between Essix and Control group showed significant difference at all time intervals. Comparison of optical density within the cold cure group at different time intervals shows no significant difference between any pair comparison. Comparison of optical density within the control group at different time intervals showed significant difference between 1 month vs 4 months ,1month vs 8 month ,1 month vs 1 year, 2 month vs 4 months , 2 month vs 8 months ,2 month vs 1 year, 4 month vs 6 months, 4 months vs 1 year, 6 month vs 8 months, 6 months vs 1 year and 8 months vs 1year samples.

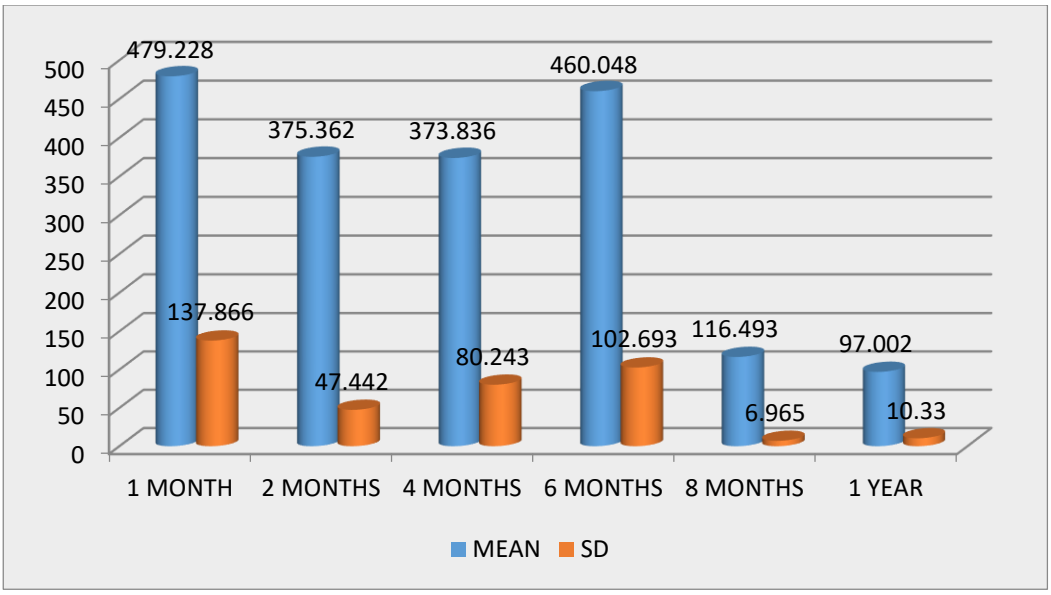
GRAPHS

GRAPH 1- CELL VIABILITY - COLD CURE



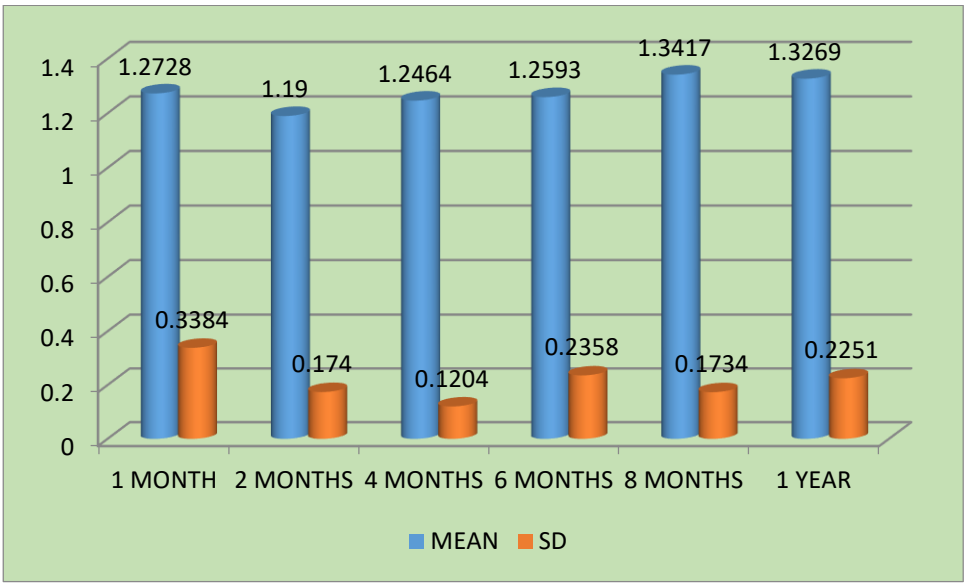
Graph 1: The mean cell viability values in the cold cure group.

GRAPH 2- CELL VIABILITY - ESSIX



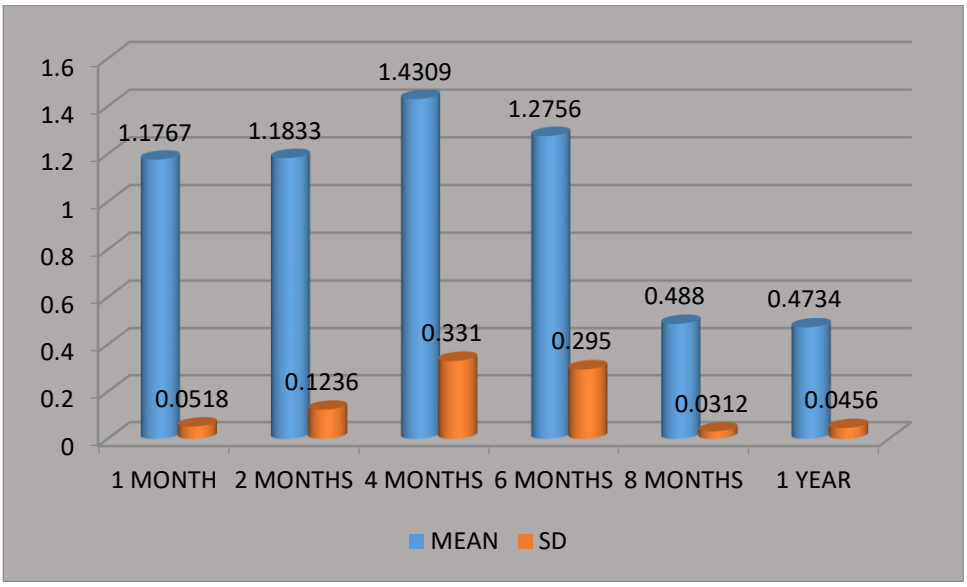
Graph 2: The mean cell viability values in the Essix group.

GRAPH 3-OPTICAL DENSITY-COLD CURE



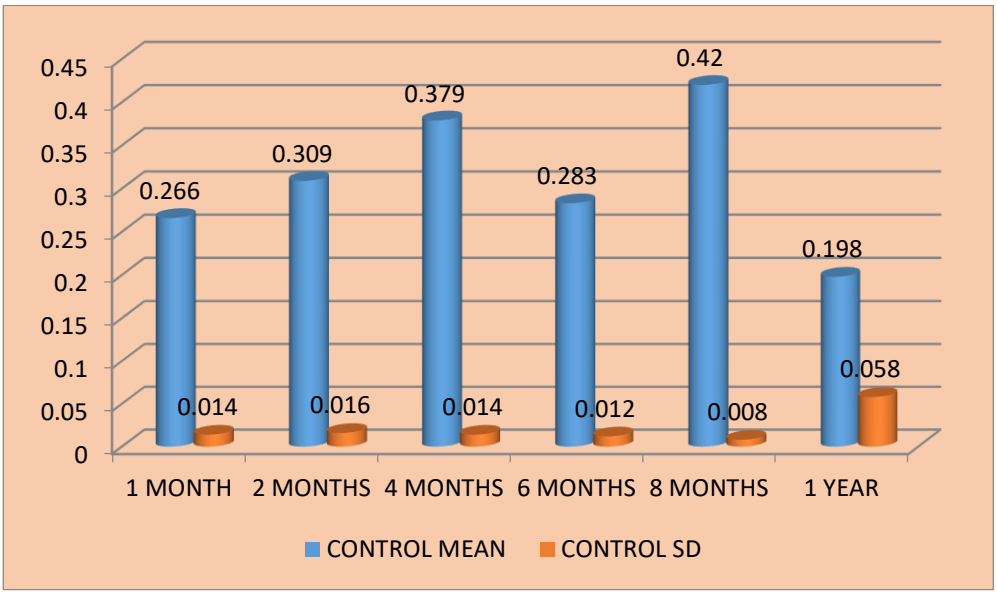
Graph 3 : The mean optical Density Values in the Cold Cure Group.

GRAPH 4- OPTICAL DENSITY- ESSIX



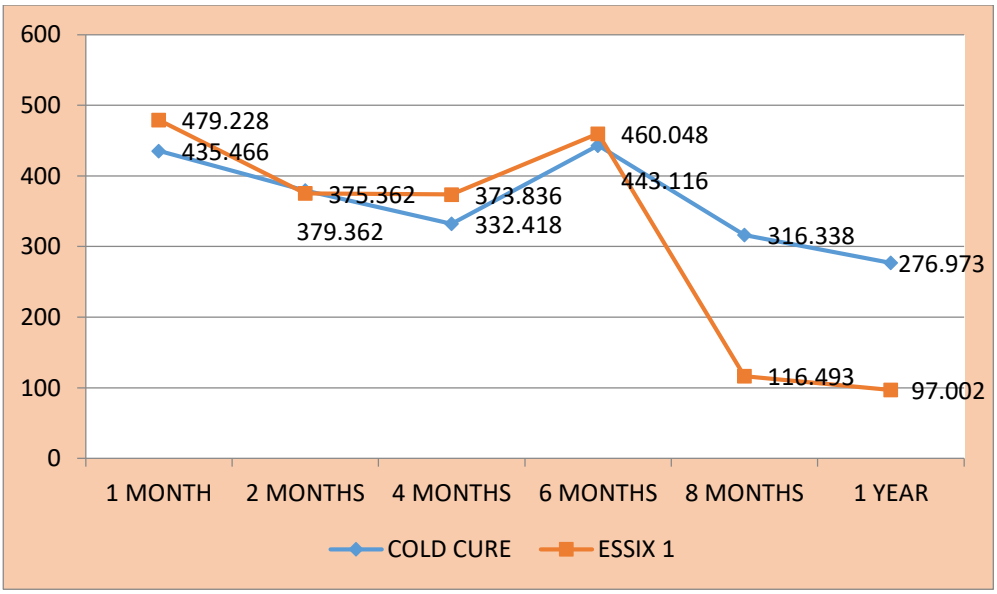
Graph 4: The mean optical Density Values in the Essix Group.

GRAPH 5-OPTICAL DENSITY- CONTROL



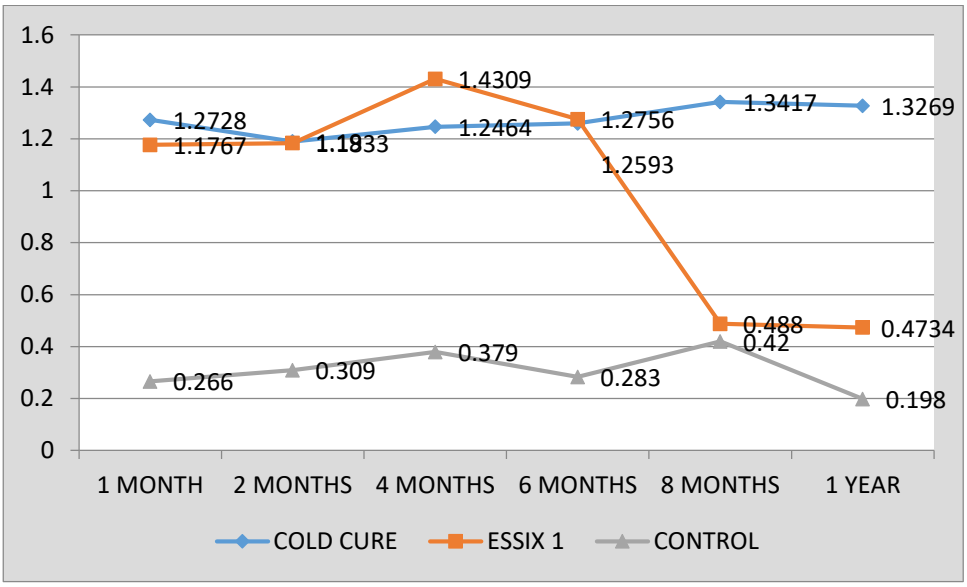
Graph 5: The mean optical Density Values in the Control Group.

GRAPH 6- COMPARISON OF CELL VIABILITY (MEAN VALUES)



Graph 6: The comparison of mean values of cell viability.

GRAPH 7- OPTICAL DENSITY COMPARISON –MEAN VALUES



Graph 7: The comparison of mean optical density values.

DISCUSSION

DISCUSSION

Essix retainer and Hawley's retainer are two commonly prescribed appliances in orthodontic treatment after fixed appliance therapy. The Essix retainer, being more aesthetic, have an increasing popularity among patients. As these appliances are used to prevent relapse after treatment and are worn by the patients for a longer duration of time, the effects of these materials on oral epithelium is very critical.

The Hawley's retainer is made of an acrylic resin composed of high molecular weight polymers (Polymethyl Methacrylate) which polymerizes in an addition reaction. Although there are thermopolymerized and autopolymerized acrylic resins, autopolymerized resins remain the most popular material for use in orthodontics because of their low cost and ease of use. The Essix retainer is made of a polyurethane material, a product of 4, 40 -methylene diphenyl diisocyanate and 1,6-hexanedial precursors, the same material which is used in the fabrication of Invisalign appliances. When isocyanates come into contact with tissues, they rapidly bind to proteins and other biomacromolecules. This reaction forms immunogenic haptens that can lead to sensitization in humans.[73-75]

Several studies were published on the cytotoxicity of acrylates, but in general most of them reported testing of prosthodontic materials. The studies in the scientific literature on the cytotoxicity of Essix retainer are very limited. This study was done to evaluate and compare the cytotoxicity of Hawley's (cold cure) and Essix retainers in an in-vitro environment. The study was designed with a longer time frame as the retainers are intended to be used for a longer duration.

L929 fibroblast cell lines were used in this study. Schmalz and Browne[76] stated that permanent cell lines should be used in a standard assay for toxicity screening since they are well defined and generally available for good reproducibility of results. They are rather simple replicating systems. According to the authors, this is the philosophy of ISO 10993 part 5 on the standardization of cell culture experiments. For these reasons, established cell lines were used in the present study.[76]

MTT assay was used for cytotoxicity testing. This assay quantifies the mitochondrial succinate dehydrogenase enzyme activity and measures the conversion of the water-soluble tetrazolium salts into insoluble purple formazan crystals by

spectrophotometry. The optical density is measured at two different wavelengths, the measurement (490 nm) and reference wavelength (655 nm) which was done on a microplate reader. The optical density values are proportional to the number of viable cells. As the optical density increases, the cell viability also increases indicating lower level of toxicity. Decreased optical density values are associated with decreased number of viable cells. Percentage of viable cells is calculated from the optical density values. This test is an excellent marker of cell survival because it evaluates cellular respiratory activity. The experiment was done in triplicates for each test group and the average was taken.

Sheridan et al[30] evaluated the cytotoxic effect of heat-activated, chemically-activated and microwave-activated acrylic resins on gingival fibroblasts and reported that the greatest cytotoxic effect was produced by the chemically activated acrylic resins. They stated that the presence and the amount of free monomers in the resin is one of the most important factors in inducing the cytotoxic reaction and greater residual monomer has been shown to cause greater cytotoxicity.

In the current study, we have found that the cells treated with self cure acrylic resin eluate were viable during all the time intervals tested indicating no cytotoxicity associated with them.

Rose et al[35] tested autopolymerized and thermopolymerized orthodontic acrylic resins and evaluated the residual monomer and cytotoxicity testing on established cultured fibroblasts. They stated that all orthodontic materials had a low cytotoxicity and that thermopolymerized resins were not considered cytotoxic

According to Hensten Pettersen and Wictorin[23] polymerization influences cytotoxicity. Their studies revealed lower cell growth in self-curing resins in comparison to heat-curing ones. Gonçalves et al[49] also assessed the cytotoxicity of acrylic resins for orthodontic purposes within 24 and 48 hours and reported that there was less cell viability after 24 hours. Our study results are in disagreement to these findings. This difference can be explained by the cell type used, the time duration and the material used for elution. Gonsalves et al[49] used epithelial cells instead of fibroblasts. Moreover in their study, the sample eluate was done with culture medium whereas in our study artificial saliva was used and the samples were tested after a long time duration contrary to the 24 and 48 hour testing done in the above studies.

Eliades et al[53] reported no cytotoxic or estrogenic activity of Invisalign appliances in which they used a standard model for the assessment of estrogenicity of materials.

Shaima R Al Naqbi et al[72] tested the cytotoxic and estrogenic behaviour of both as-received and retrieved Vivera retainers and reported that there is no significant estrogenic activity after the incubation of both groups of these retainers in normal saline for two weeks at body temperature. This is in accord with our study results in which we found that the optical density values were within the normal range for all the samples at all time intervals. Although the optical density values for the 8 months and 1 year samples showed a slight decrease; the average percentage cell viability calculated from these values fell within the normal range.

Thyagaseely Premaraj et al[14] reported that exposure to Invisalign plastic caused changes in viability, membrane permeability and adhesion of epithelial cells in a saline-solution environment. However, when exposed to artificial saliva, the cells behaved normally or even showed increased cell-to-cell contact. They proposed that saliva might play an important role in maintaining epithelial cell integrity.

The cytotoxic properties of thermoplastic materials appear to be influenced by polymer composition and structure, as well as by processing and environmental factors such as temperature, humidity, pressure and thermal history. Jorge et al[77] in a 2003 review article, reported differences in cytotoxic effects of polymeric materials to various factors, including chemical composition of the material and the polymerization methods that were used. Specifically, the polymer-to-monomer ratio, storage time in water, polymerization cycle and the method of polymerization influences the reactivity of the polymer. Polymeric materials, particularly amorphous plastics that include Invisalign, demonstrate high water sorption rates, which permit long-term leaching of unreacted components that in turn remain in contact with cells.

In the current study, the MTT assay values revealed that the percentage cell viability of most of the test samples were more than that of the control (artificial saliva); i.e more than 100 %. Although there was a statistically significant difference in the optical density values for the 8 months and 1 year samples of Essix retainer, the

cell viability values were well within the normal range which indicated that all of the test materials were biocompatible.

The cell viability percentage of above 100 % for both the Essix and cold cure material cannot be interpreted as cell proliferation. This can be due to multiple reasons. One of the major reason for such variation is the stimulation by the treatment which is scientifically termed as 'hormesis'.[78] MTT is prone to compounds interferring with energy metabolism (e.g. uncouplers) which can increase MTT metabolism to up to 200 % baseline activity. Another possibility is the direct chemical reduction of MTT by Essix/Cold cure eluates.[79]

MTT assay is far superior to the previously used dye exclusion methods in terms of sensitivity and reproducibility. It is simple to perform and is also a safe method for testing.[80] Although this has been the most widely used colorimetric assay for cytotoxicity testing, there are many limitations associated with this assay.[81] The reduction of the substrate may be impacted by changes in the intracellular metabolic activity without any direct effect on overall cell viability. The organic solvents used for solubilizing may precipitate proteins from some serum-supplemented culture medium, and this may cause light scattering. [82] Some of the assay conditions might affect the chemical or enzymatic reduction of MTT which will be projected as increased background absorbance values and cell proliferation. Many of the chemicals, particularly the reducing compounds can itself interfere with the MTT assay leading to non-enzymatic reduction of the MTT to purple coloured formazan. Moreover, the long-term exposure of MTT assay reagent to light and increased pH of the culture medium could result in the production of more formazan and hence higher background absorbance readings.[84,85]

This test does not completely represent the cytotoxic properties of Essix and Hawley's retainer in a clinical set up. The in-vitro aging of a material is substantially different from that of in-vivo conditions.[86] In this study we obtained eluates under static conditions, which is not the case in an intraoral environment where the patient often removes the appliance from the mouth. Moreover, in intraoral environment these retainers can be exposed to hot food and liquid which creates a transient heat shock and might influence the biocompatibility.

More than one assay for cytotoxicity testing can increase the reliability of the results obtained in the in-vitro studies. Other cell viability assay methods with greater detection sensitivity and those that record the data repeatedly in real time and more effectively in 3D culture can provide a more holistic view on the biocompatibility of Essix and Hawley's retainers.

The limitations of this study:

1. As this study was done in an in vitro condition, the results cannot be extrapolated for the general population. Intraoral environment is dynamic and cannot be simulated completely in vitro where the environment is more static. As the polyurethanes are not inert materials, they are highly affected by heat, moisture and prolonged contact with salivary enzymes.
2. Owing to difficulties with the collection and storage of natural saliva and the chances of cross contamination, artificial saliva was used in this study as the control group.
3. The MTT assay data does not reflect what is happening in the cells in real time. The amount of signal generated is dependent on several parameters: including the concentration of MTT, the length of the incubation period, the number of viable cells and their metabolic activity.

CONCLUSION

CONCLUSION

After cytotoxicity testing with MTT assay, it was noticed that the Hawley's retainer samples do not show any decrease in the percentage cell viability at any time intervals. The samples of Essix retainer tested at 8 months and 1 year showed a slight decrease in the percentage cell viability. Though this decrease was found to be statistically significant compared to the other groups, these values were also well within the acceptable range of cell viability.

All the samples of Hawley's retainer showed a cell viability above 100%. All the samples of Essix retainer except those at 8 months and 1 year time period showed a cell viability more than 100%. This could be due to direct reduction of the MTT reagent non enzymatically. Whether these values above 100% have any significance will have to be confirmed with a second cytotoxicity assay. The MTT assay alone cannot be confirmatory for any in vitro study.

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ANNEXURES

Annexure I- Results of The 1 month samples

Samples	**Optical Density Values (At 490 Nm, At 655 Nm & The Calculated Difference)**		
Control	0.3838	0.1165	0.2673
Control	0.3567	0.1101	0.2466
Control	0.3681	0.1215	0.2548
Control	0.3372	0.0717	0.2655
Control	0.3584	0.074	0.2844
Control	0.3217	0.052	0.2697
Control	0.3829	0.1215	0.2614
Control	0.3361	0.0886	0.2475
Control	0.3745	0.0891	0.2854
Control	0.3694	0.0899	0.2795

Samples	Optical Density At 490 Nm			Optical Density At 655 Nm		
Cold cure	1.3652	1.2546	1.5376	0.3124	0.2134	0.1975
Cold cure	1.4617	1.036	0.9263	0.2717	0.1958	0.1816
Cold cure	1.3359	1.5415	2.0961	0.228	0.2466	0.2953
Cold cure	1.9793	2.0416	2.4857	0.3355	0.3505	0.4285
Cold cure	1.5643	1.2496	1.4532	0.1094	0.1165	0.3202
Cold cure	1.0765	1.1967	1.3076	0.2865	0.1986	0.1567
Cold cure	2.0134	1.8564	1.9065	0.265	0.1875	0.1439
Cold cure	1.3452	1.2678	1.1763	0.2745	0.1945	0.167
Cold cure	1.7654	1.8123	1.2546	0.0984	0.1258	0.0962
Cold cure	1.8745	1.6546	1.2345	0.0887	0.0879	0.0431
Essix	1.2835	1.1936	1.1543	0.4999	0.5012	0.3192
Essix	1.3934	1.4935	1.6432	0.3817	0.1224	0.2218
Essix	1.1271	1.1652	1.1477	0.2038	0.2170	0.2181
Essix	0.3312	0.7341	0.6529	0.0872	0.1502	0.1365
Essix	1.0732	0.8785	1.0792	0.1945	0.172	0.2022
Essix	1.3546	1.6578	1.3456	0.0674	0.1568	0.1893
Essix	2.1065	1.9376	1.6745	0.2657	0.2237	0.2564
Essix	1.9856	1.7634	1.8546	0.1875	0.1546	0.1765
Essix	1.2637	1.0548	1.2894	0.1786	0.2509	0.2195
Essix	1.7649	1.8346	1.4453	0.16	0.1496	0.1549

Samples	Calculated Difference of Optical Density		
Cold cure	1.0528	1.0412	1.3401
Cold cure	1.19	0.8402	0.7446
Cold cure	1.1079	1.2949	1.8008
Cold cure	1.6439	1.6911	2.0572
Cold cure	1.4549	1.1331	1.133
Cold cure	0.79	0.9981	1.1509
Cold cure	1.7484	1.6689	1.7626
Cold cure	1.0707	1.0733	1.0093
Cold cure	1.667	1.6865	1.1584
Cold cure	1.7858	0.2467	0.1777
Essix	0.7836	0.6924	0.8351
Essix	1.0117	1.3711	1.4214
Essix	0.9233	0.9480	0.9296
Essix	0.244	0.5838	0.5164
Essix	0.8787	0.7066	0.877
Essix	0.6806	1.501	1.1563
Essix	1.8408	1.7139	1.4181
Essix	1.7981	1.6088	1.6781
Essix	1.0851	0.8039	1.0699
Essix	1.6049	1.685	1.2904

Samples	Percentage Cell Viability		
Cold cure	395.47725	391.119793	503.3996
Cold cure	447.01554	315.615494	279.704
Cold cure	416.17521	486.420473	676.4584
Cold cure	617.52002	635.250367	772.7734
Cold cure	546.52342	425.641411	425.6038
Cold cure	296.7582	374.929567	432.3279
Cold cure	656.77473	626.911085	662.1089
Cold cure	402.20127	403.177942	379.1368
Cold cure	626.19736	633.522407	435.1452
Cold cure	670.82379	92.6712008	66.75181
Essix	294.35408	260.095413	313.6997
Essix	380.03832	515.044514	533.9394
Essix	346.83146	356.147391	349.198
Essix	91.656964	219.300559	193.9822
Essix	255.66282	563.840577	434.3563
Essix	255.66282	563.840577	434.3563
Essix	691.48417	643.815033	532.6997
Essix	675.4442	604.334924	630.367
Essix	407.61053	301.97964	401.9008
Essix	602.86991	632.958942	484.7301

Annexure II- Results of the 2 months samples

Samples	**Optical Density Values (At 490 Nm, At 655 Nm & The Calculated Difference)**		
Control	0.3567	0.04	0.3167
Control	0.3688	0.0415	0.3273
Control	0.3142	0.0145	0.2997
Control	0.3317	0.0132	0.3185
Control	0.3148	0.0292	0.2856
Control	0.3491	0.0276	0.3215
Control	0.3315	0.0198	0.3117
Control	0.3485	0.0471	0.3014
Control	0.3394	0.0117	0.3277
Control	0.4512	0.1686	0.2826

Samples	Optical Density At 490 Nm			Optical Density At 655 Nm		
Cold cure	1.4867	1.2316	1.1321	0.4321	0.2543	0.2097
Cold cure	1.3865	1.4097	1.0872	0.3054	0.3984	0.1093
Cold cure	1.6543	1.3098	1.2743	0.4963	0.2874	0.1086
Cold cure	2.012	1.6734	1.7609	0.4764	0.3876	0.3764
Cold cure	1.4239	1.2496	1.329	0.1342	0.2986	0.1498
Cold cure	2.0543	1.6532	1.8794	0.4012	0.293	0.3945
Cold cure	1.3454	1.4095	1.2765	0.1397	0.1756	0.2895
Cold cure	1.1965	1.2764	1.1432	0.2056	0.1876	0.167
Cold cure	1.7654	1.6231	1.5342	0.3428	0.2341	0.2986
Cold cure	1.2349	1.2675	1.2454	0.0887	0.0845	0.0398
Essix	1.3654	1.3096	1.2453	0.1954	0.2015	0.1567
Essix	1.6532	1.4567	1.469	0.3097	0.4985	0.2349
Essix	1.1675	1.0983	1.1873	0.0342	0.1892	0.2784
Essix	1.4053	1.3074	1.6732	0.1982	0.0659	0.2765
Essix	1.0486	1.1732	1.2094	0.0569	0.1061	0.184
Essix	1.5073	1.4376	1.3675	0.2931	0.1891	0.1681
Essix	1.8453	1.5697	1.3847	0.3867	0.2965	0.2564
Essix	1.5903	1.4096	1.3965	0.2046	0.1546	0.1765
Essix	1.3056	1.3215	1.2056	0.1483	0.2832	0.1276
Essix	1.6843	1.5943	1.4476	0.2841	0.1496	0.2316

Samples	Calculated Difference of Optical Density		
Cold cure	1.0546	0.9773	0.9224
Cold cure	1.0811	1.0113	0.9779
Cold cure	1.158	1.0224	1.1657
Cold cure	1.5356	1.2858	1.3845
Cold cure	1.4549	0.951	1.1792
Cold cure	1.6531	1.3602	1.4849
Cold cure	1.2057	1.2339	0.987
Cold cure	0.9909	1.0888	0.9762
Cold cure	1.4226	1.389	1.2346
Cold cure	1.1462	1.183	1.2056
Essix	1.17	1.1081	1.0886
Essix	1.3435	0.9582	1.2341
Essix	1.1333	0.9091	0.9089
Essix	1.2071	1.2415	1.3967
Essix	0.9917	1.0671	1.0254
Essix	1.2142	1.2485	1.1994
Essix	1.4586	1.2732	1.1283
Essix	1.3857	1.255	1.22
Essix	1.1573	1.0383	1.078
Essix	1.4002	1.4447	1.216

Samples	Percentage Cell Viability		
Cold cure	340.9965402	316.0022	298.2507
Cold cure	349.5651049	326.99583	316.1962
Cold cure	374.4301096	330.58493	376.9198
Cold cure	496.5240728	415.75323	447.6671
Cold cure	470.4303683	307.4983	381.285
Cold cure	534.5167653	439.80987	480.1306
Cold cure	389.853526	398.97177	319.1386
Cold cure	320.3996508	352.05484	315.6465
Cold cure	459.9864196	449.12213	399.1981
Cold cure	370.6146733	382.51366	389.8212
Essix	378.3102144	358.29534	351.9902
Essix	434.4100624	309.82637	399.0364
Essix	366.4435606	293.95027	293.8856
Essix	390.3062049	401.42917	451.6119
Essix	320.6583244	345.03832	331.555
Essix	392.6019336	403.69257	387.8165
Essix	471.6267339	411.67912	364.8269
Essix	448.0550975	405.79429	394.4773
Essix	374.2037702	348.56274	348.5627
Essix	452.7435574	467.13228	393.1839

Annexure III- Results of the 4 months samples

Samples	**Optical Density Values (At 490 Nm, At 655 Nm & The Calculated Difference)**		
Control	0.4122	0.0209	0.3913
Control	0.3914	0.0071	0.3843
Control	0.5137	0.1027	0.411
Control	0.3874	0.0203	0.3671
Control	0.3965	0.0206	0.3759
Control	0.3867	0.0296	0.3571
Control	0.4055	0.036	0.3695
Control	0.4158	0.0403	0.3755
Control	0.4017	0.0206	0.3811
Control	0.4512	0.0724	0.3788

Samples	Optical Density At 490 Nm			Optical Density At 655 Nm		
Cold cure	1.5732	1.3186	1.5476	0.4454	0.3749	0.1981
Cold cure	1.4523	1.4097	1.2765	0.1094	0.1354	0.0902
Cold cure	1.5638	1.3496	1.2319	0.2987	0.1902	0.1943
Cold cure	1.5762	1.6537	1.7731	0.2906	0.4396	0.4314
Cold cure	1.6279	1.5142	1.4269	0.1497	0.1685	0.1504
Cold cure	1.7254	1.5326	1.6257	0.1023	0.095	0.2495
Cold cure	1.5248	1.4279	1.4327	0.2405	0.2325	0.2196
Cold cure	1.2436	1.3525	1.4524	0.26	0.3108	0.3255
Cold cure	1.5902	1.6231	1.4271	0.3028	0.306	0.1925
Cold cure	1.3421	1.3782	1.5978	0.1629	0.1186	0.3922
Essix	1.5239	1.4275	1.5238	0.4494	0.2411	0.0662
Essix	1.6274	1.7123	1.6236	0.1642	0.145	0.164
Essix	1.5289	1.4312	1.5874	0.1314	0.1518	0.3013
Essix	1.6277	1.8432	2.5729	0.1685	0.2754	1.2538
Essix	1.5328	1.7254	1.5367	0.3861	0.44	0.4798
Essix	1.7235	1.7496	1.5126	0.4039	0.464	0.2975
Essix	1.7128	1.8176	1.6238	0.6966	0.5102	0.4344
Essix	2.7265	2.4187	2.4067	0.2701	0.2644	0.19
Essix	2.0162	2.0451	1.9032	0.2598	0.503	0.3314
Essix	1.6843	1.5413	1.5341	0.3524	0.2448	0.2722

Samples	Calculated Difference of Optical Density		
Cold cure	1.1278	0.9437	1.3495
Cold cure	1.3429	1.2743	1.1863
Cold cure	1.2651	1.1594	1.0376
Cold cure	1.2856	1.2141	1.3417
Cold cure	1.4782	1.3457	1.2765
Cold cure	1.6231	1.4376	1.3762
Cold cure	1.2843	1.1954	1.2131
Cold cure	0.9836	1.0417	1.1269
Cold cure	1.2874	1.3171	1.2346
Cold cure	1.1792	1.2596	1.2056
Essix	1.0745	1.1864	1.4576
Essix	1.4632	1.5673	1.4596
Essix	1.3975	1.2794	1.2861
Essix	1.4592	1.5678	1.3191
Essix	1.1467	1.2854	1.0569
Essix	1.3196	1.2856	1.2151
Essix	1.0162	1.3074	1.1894
Essix	2.4564	2.1543	2.2167
Essix	1.7564	1.5421	1.5718
Essix	1.3319	1.2965	1.2619

Samples	Percentage Cell Viability		
Cold cure	297.4469881	248.89229	355.9183
Cold cure	354.1776559	336.08503	312.8758
Cold cure	333.6586138	305.7812	273.6576
Cold cure	339.0653022	320.20783	353.8612
Cold cure	389.8617998	354.91613	336.6653
Cold cure	428.0778563	379.15392	362.9602
Cold cure	338.7224391	315.27587	319.9441
Cold cure	259.4155502	274.7389	297.2096
Cold cure	339.5400359	347.37314	325.6145
Cold cure	311.0032704	332.20804	317.966
Essix	283.3895981	312.9022	384.4287
Essix	385.9056863	413.36111	384.9562
Essix	368.5779091	337.43011	339.1972
Essix	384.8507227	413.49298	347.9006
Essix	302.4316911	339.01255	278.7478
Essix	348.0324929	339.0653	320.4716
Essix	268.0135035	344.81485	313.6934
Essix	647.8531491	568.17702	584.6345
Essix	463.2345184	414.54795	414.5479
Essix	351.276506	341.94008	332.8146

Annexure IV- Results of the 6 months samples

Samples	**Optical Density Values (At 490 Nm, At 655 Nm & The Calculated Difference)**		
Control	0.3214	0.0417	0.2797
Control	0.3341	0.0459	0.2882
Control	0.2954	0.0238	0.2716
Control	0.3231	0.0247	0.2984
Control	0.3562	0.0538	0.2979
Control	0.2843	0.0191	0.2652
Control	0.2984	0.0319	0.2665
Control	0.3104	0.0305	0.2799
Control	0.3016	0.0022	0.2994
Control	0.2997	0.0122	0.2875

Samples	Optical Density At 490 Nm			Optical Density At 655 Nm		
Cold cure	1.6782	1.5296	1.4965	0.444	0.2562	0.3418
Cold cure	1.7563	1.3782	1.4026	0.3735	0.364	0.317
Cold cure	1.3826	1.2874	1.3122	0.4482	0.3656	0.295
Cold cure	1.5305	1.4327	1.69342	0.2661	0.2689	0.38662
Cold cure	2.0548	1.9354	1.7838	0.3614	0.3355	0.3777
Cold cure	1.2393	1.1056	1.2638	0.4154	0.1801	0.4403
Cold cure	1.3896	1.4283	1.5474	0.2258	0.2548	0.1749
Cold cure	1.8335	1.73801	2.0143	0.1815	0.37321	0.2711
Cold cure	1.6945	1.6294	1.7155	0.4124	0.424	0.2206
Cold cure	1.8259	1.7926	1.6485	0.4472	0.3087	0.2094
Essix	1.4238	1.2554	1.2397	0.2956	0.2208	0.2938
Essix	1.6399	1.7325	1.6235	0.3452	0.3836	0.2492
Essix	1.3947	1.2846	1.2634	0.3202	0.2197	0.0899
Essix	1.1937	1.0453	1.2045	0.3003	0.3104	0.3704
Essix	1.0345	1.1752	1.4529	0.1918	0.1821	0.1782
Essix	1.8452	1.8672	1.9243	0.517	0.511	0.5117
Essix	1.5932	1.4744	1.3091	0.1949	0.1834	0.1453
Essix	2.2847	2.1674	2.0345	0.5527	0.5327	0.3198
Essix	1.6616	1.6732	1.7456	0.353	0.461	0.353
Essix	2.1212	2.1846	1.9037	0.2864	0.4038	0.2082

Samples	Calculated Difference of Optical Density		
Cold cure	1.2342	1.2734	1.1547
Cold cure	1.3828	1.0142	1.0856
Cold cure	0.9344	0.9218	1.0172
Cold cure	1.2644	1.1638	1.3068
Cold cure	1.6934	1.5999	1.4061
Cold cure	0.8239	0.9255	0.8235
Cold cure	1.1638	1.1735	1.3725
Cold cure	1.652	1.3648	1.7432
Cold cure	1.2821	1.2054	1.4949
Cold cure	1.3787	1.4839	1.4391
Essix	1.1282	1.0346	0.9459
Essix	1.2947	1.3489	1.3743
Essix	1.0745	1.0649	1.1735
Essix	0.8934	0.7349	0.8341
Essix	0.8427	0.9931	1.2747
Essix	1.3282	1.3562	1.4126
Essix	1.3983	1.291	1.1638
Essix	1.732	1.6347	1.7147
Essix	1.3086	1.2122	1.3926
Essix	1.8348	1.7808	1.6955

Samples	Percentage Cell Viability		
Cold cure	435.451434	449.28201	407.4022
Cold cure	487.880605	357.830858	383.0223
Cold cure	329.675758	325.230216	358.8893
Cold cure	446.106622	410.61285	461.0662
Cold cure	597.466747	564.478002	496.1013
Cold cure	290.689059	326.535653	290.5479
Cold cure	410.61285	414.035212	484.2466
Cold cure	582.859965	481.529831	615.0372
Cold cure	452.351551	425.290195	527.4318
Cold cure	486.43404	523.550789	507.7444
Essix	398.052429	365.028402	333.7332
Essix	456.797093	475.91998	484.8816
Essix	379.105952	375.718872	414.0352
Essix	315.210105	259.288008	294.2878
Essix	297.32209	350.386339	449.7407
Essix	468.61659	478.495572	498.3947
Essix	493.349328	455.491656	410.6128
Essix	611.08563	576.756166	604.9818
Essix	461.701302	427.689377	491.3382
Essix	647.355608	628.303285	598.2077

Annexure V- Results of the 8 months samples

Samples	**Optical Density Values (At 490 Nm, At 655 Nm & The Calculated Difference)**		
Control	0.6511	0.2377	0.4124
Control	0.5485	0.1254	0.4231
Control	0.5598	0.1553	0.4045
Control	0.6176	0.1789	0.4387
Control	0.7843	0.3665	0.4178
Control	0.6718	0.2483	0.4235
Control	0.6251	0.2073	0.4178
Control	0.8421	0.4187	0.4234
Control	0.9151	0.4884	0.4267
Control	0.7399	0.3213	0.4186

Samples	Optical Density At 490 Nm			Optical Density At 655 Nm		
Cold cure	1.7623	1.5074	1.6937	0.4205	0.2331	0.4647
Cold cure	1.3115	1.2957	1.4852	0.0334	-0.1975	0.2467
Cold cure	1.6401	1.5991	1.5106	0.4669	0.4352	0.3569
Cold cure	1.6678	1.4751	1.5577	0.2532	0.1545	0.247
Cold cure	1.8933	1.7766	1.6051	0.4174	0.2877	0.2128
Cold cure	1.6046	1.5814	1.5905	0.6462	0.3888	0.3829
Cold cure	1.3822	1.4044	1.4167	0.0887	0.0636	0.0346
Cold cure	1.5789	1.6943	1.5019	0.2805	0.5246	0.2776
Cold cure	2.1596	2.2937	2.0138	0.4161	0.605	0.2316
Cold cure	1.5295	1.6883	1.4922	0.163	0.2044	0.1217
Essix	0.6754	0.8056	0.7135	0.2576	0.2933	0.2374
Essix	0.8181	0.6152	0.6698	0.317	0.1179	0.1937
Essix	0.7684	0.6015	0.6178	0.3117	0.1336	0.1721
Essix	0.7894	0.8736	0.7685	0.2783	0.3754	0.1671
Essix	0.6614	0.5984	0.6793	0.1263	0.1067	0.2215
Essix	0.8864	0.6531	0.7532	0.4223	0.2186	0.2743
Essix	0.8716	0.8145	0.6753	0.3609	0.3364	0.163
Essix	0.7769	0.6193	0.6576	0.359	0.1099	0.1404
Essix	0.5783	0.4987	0.7864	0.0712	0.0673	0.3709
Essix	0.6743	0.7717	0.6171	0.0728	0.2542	0.1187

Samples	Calculated Difference of Optical Density		
Cold cure	1.3418	1.2743	1.229
Cold cure	1.2781	1.4932	1.2385
Cold cure	1.1732	1.1639	1.1537
Cold cure	1.4146	1.3206	1.3107
Cold cure	1.4759	1.4889	1.3923
Cold cure	0.9584	1.1926	1.2076
Cold cure	1.2935	1.3408	1.3821
Cold cure	1.2984	1.1697	1.2243
Cold cure	1.7435	1.6887	1.7822
Cold cure	1.3665	1.4839	1.3705
Essix	0.4178	0.5123	0.4761
Essix	0.5011	0.4973	0.4761
Essix	0.4567	0.4679	0.4457
Essix	0.5111	0.4982	0.6014
Essix	0.5351	0.4917	0.4578
Essix	0.4641	0.4345	0.4789
Essix	0.5107	0.4781	0.5123
Essix	0.4179	0.5094	0.5172
Essix	0.5071	0.4314	0.4155
Essix	0.6015	0.5175	0.4984

Samples	Percentage Cell Viability		
Cold cure	318.906714	302.863933	292.0974
Cold cure	303.767083	354.890077	294.3553
Cold cure	278.835413	276.625074	274.2008
Cold cure	336.20915	313.868093	311.5152
Cold cure	350.778372	353.868093	330.9091
Cold cure	227.78372	283.446227	287.0113
Cold cure	307.427213	318.669043	328.4848
Cold cure	308.5918	278.003565	290.9804
Cold cure	414.379085	401.354724	423.5769
Cold cure	324.777184	352.679739	325.7279
Essix	99.2988711	121.758764	113.1551
Essix	119.096851	118.193702	113.1551
Essix	108.544266	111.206179	105.9299
Essix	121.473559	118.407605	142.9352
Essix	127.177659	116.862745	108.8057
Essix	110.30303	103.267974	113.8206
Essix	121.378491	113.630422	121.7588
Essix	99.3226381	121.069519	122.9234
Essix	120.522876	102.531194	98.75223
Essix	142.959002	122.994652	118.4551

Annexure VI- Results of The 1 year samples

Samples	**Optical Density Values (At 490 Nm, At 655 Nm & The Calculated Difference)**		
Control	0.6783	0.2014	0.4769
Control	0.7431	0.2514	0.4917
Control	0.7134	0.2703	0.4431
Control	0.6984	0.1967	0.5017
Control	0.6655	0.1466	0.5189
Control	0.6275	0.1600	0.4675
Control	0.7547	0.3031	0.4516
Control	0.6165	0.1291	0.4874
Control	0.6346	0.1458	0.4888
Control	0.6752	0.1767	0.4985

Samples	Optical Density At 490 Nm			Optical Density At 655 Nm		
Cold cure	2.1644	2.0247	1.8745	0.7062	0.462	0.2484
Cold cure	1.2679	1.3565	1.4852	0.3353	0.5202	0.471
Cold cure	1.8779	1.9921	1.8172	0.5351	0.5664	0.5315
Cold cure	1.3377	1.4156	1.5678	0.2319	0.1217	0.3402
Cold cure	1.5674	1.8734	1.6395	0.2532	0.3845	0.2472
Cold cure	1.4397	1.5814	1.5932	0.4813	0.3888	0.3856
Cold cure	1.6733	1.5893	1.5159	0.3798	0.2485	0.1338
Cold cure	1.8432	1.9779	1.7491	0.5448	0.8082	0.5248
Cold cure	1.8688	1.8369	1.8764	0.1253	0.1482	0.0942
Cold cure	1.5574	1.6764	1.7938	0.1909	0.1925	0.4233
Essix	0.9341	0.8864	0.8945	0.4467	0.4296	0.3926
Essix	0.9168	1.1452	0.9654	0.4272	0.7347	0.527
Essix	0.8539	0.9156	0.9741	0.3361	0.3789	0.4226
Essix	0.8564	0.6985	0.5864	0.1833	0.1939	0.1012
Essix	0.6683	0.7396	0.7784	0.2918	0.3411	0.3426
Essix	0.7828	0.6743	0.7564	0.3072	0.2098	0.329
Essix	0.8636	0.7641	0.6743	0.3282	0.2364	0.2392
Essix	0.7523	0.8146	0.7181	0.3267	0.3079	0.2716
Essix	0.8356	0.6654	0.5572	0.4134	0.2339	0.1011
Essix	0.7321	0.6784	0.7694	0.2897	0.2131	0.292

Samples	Calculated Difference of Optical Density		
Cold cure	1.4582	1.5627	1.6261
Cold cure	0.9326	0.8363	1.0142
Cold cure	1.3428	1.4257	1.2857
Cold cure	1.1058	1.2939	1.2276
Cold cure	1.3142	1.4889	1.3923
Cold cure	0.9584	1.1926	1.2076
Cold cure	1.2935	1.3408	1.3821
Cold cure	1.2984	1.1697	1.2243
Cold cure	1.7435	1.6887	1.7822
Cold cure	1.3665	1.4839	1.3705
Essix	0.4874	0.4568	0.5019
Essix	0.4896	0.4105	0.4384
Essix	0.5178	0.5367	0.5515
Essix	0.6731	0.5046	0.4852
Essix	0.3765	0.3985	0.4358
Essix	0.4756	0.4645	0.4274
Essix	0.5354	0.5277	0.4351
Essix	0.4256	0.5067	0.4465
Essix	0.4222	0.4315	0.4561
Essix	0.4424	0.4653	0.4774

Samples	Percentage Cell Viability		
Cold cure	302.148733	323.801828	336.9387
Cold cure	193.240919	173.286919	210.149
Cold cure	278.237086	295.414517	266.4056
Cold cure	229.12911	268.104681	254.3669
Cold cure	272.310976	308.509977	288.4938
Cold cure	198.586851	247.114647	250.2227
Cold cure	268.021798	277.822673	286.3803
Cold cure	269.037111	242.369615	253.6831
Cold cure	361.264789	349.909865	369.2837
Cold cure	283.147883	307.473944	283.9767
Essix	100.99252	94.6519964	103.997
Essix	101.448374	85.0583287	90.83939
Essix	107.291602	111.207808	114.2745
Essix	139.470794	104.556474	100.5367
Essix	78.0133027	82.5718489	90.30066
Essix	98.5474814	96.2474876	88.56012
Essix	110.938439	109.342948	90.15561
Essix	88.187149	104.991608	92.51777
Essix	87.4826464	89.4096683	94.50695
Essix	91.6682207	96.4132529	98.92045

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Dr. Anjitha Devaraj

LIST OF ABBREVIATIONS USED

BPA	Bisphenol A
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethyl Formamide
DMSO	Dimethyl Sulfoxide
DPBF	Dulbecco's Phosphate Buffered Saline
FBS	Fetal Bovine Serum
FDA	Food And Drug Administration Agency
GIBCO	Grand Island Biological Company
HD	1,6-hexanediol diacrylate
HGF	Human Gingival Fibroblast
ISO	International Standards Organization
MID	Methylene DiphenylDiisocyanate
MMA	Methyl Methacylate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
OD	Optical Density
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SPSS	Statistical Package For The Social Sciences
USEPA	United States Environmental Protection Agency
VFR	Vaccum Formed Retainer

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UNDER THE MANAGEMENT OF MJSCE TRUST, PUTHENCRUZ

CHELAD , KOTHAMANGALAM, ERNAKULAM DIST, KERALA - 686681

ETHICAL CLEARANCE CERTIFICATE

SGDC/152/2017/1733/2 Date:- 20-10-2017

To,

Dr. Anjitha Devaraj
St. Gregorios Dental College
Chelad, Kothamangalam

Dear Dr. Anjitha Devaraj,

Subject:- Ethics Committee Clearance Reg.

Protocol –Oral epithelial cell reaction after exposure to Essix and Hawley's retainers :- An *in vitro* comparative study

After the Institutional Ethics Committee (IEC) held on 20th of October, 2017, this study was examined and discussed. After the consideration, the committee had decided to approve and grant clearance for the aforementioned study.

The members who attended the meeting at which the protocol was discussed were:-

1. Dr.CKK Nair - Former BARC scientist
2. Dr.Ommen Aju Jacob - Dean, St. Gregorios Dental College, Chelad
3. Dr.Cinu Thomas A - Scientist, Senior Lecturer, Department of Pharmaceutical Sciences Centre for Professional and Advanced Studies
4. Rev. Fr. Shanu K. Paulose
5. Lissy Jose – Former Member Women's Welfare Association
6. Adv. Jose Aranjani - Advocate
7. Dr.Sauganth Paul - Senior Lecturer, Department of Biochemistry, St.Gregorios Dental College
8. Dr.Eapen Cherian - Secretary
9. Dr.Jain Mathew - Principal and Head of the Department, Department of Conservative Dentistry and Endodontics.
10. Dr.George Francis - Head of the Department, Department of Prosthodontics Crown & Bridge
11. Dr.Binnoy Kurian - Head of the Department, Department of Orthodontics & Dentofacial Orthopaedics

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