"COMPARATIVE EVALUATION OF CYTOTOXIC EFFECT OF EPOXY RESIN BASED, SILICONE BASED AND BIOCERAMIC BASED ENDODONTIC SEALERS ON HUMAN PERIODONTAL LIGAMENT FIBROBLASTS BY MTT ASSAY: - AN IN-VITRO STUDY"

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LIST OF ABBREVIATIONS

CO_2	Carbon dioxide
cm^2	Centimeter square
DCT	Direct Contact Test
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxy-Ribo Nucleic Acid
EDX	Energy Dispersive X-Ray
ELISA	Enzyme Linked Immuno Sorbent Assay
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
Fig	Figure
hrs	Hours
HGF	Human Gingival Fibroblasts
hPDLSCs	Human Periodontal Ligament Stem Cells
min	Minutes
ml	Milliliter

mM	Millimole
µm	Micrometer
µl	Microliter
MTA	Mineral Trioxide Aggregate
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay
nm	Nanometer
NaCl	Sodium Chloride
PBS	Phosphate Buffer Saline
PDL	Periodontal ligament fibroblasts
Sec	Seconds
SEM	Scanning Electron Microscopy
SD	Standard Deviation
SE	Standard Error

ABSTRACT

Aim

To evaluate and compare the cytotoxic effect of the Epoxy resin based, Silicone based and Bioceramic based endodontic sealers on human periodontal ligament fibroblasts by MTT assay.

Materials and Methodology –

- Cell culture of human periodontal ligament fibroblasts

Periodontal tissues excised from extracted premolar teeth indicated for orthodontic extraction was used for establishment of explant cultures and subsequent sub-cultures.

- Preparation of Test materials

Test groups made were: -

Group 1: - AH Plus (Epoxy resin based sealer)

Group 2: - GuttaFlow bioseal (Silicone based sealer)

Group 3: - Bio-C sealer (Bioceramic based sealer)

Group 4: - Negative control (No sealer)

Eighteen separate discs of each sealer using cylindrical teflon moulds (2mm diameter and 2mm in length) were made and allowed to set for 24 hrs.

- Cytotoxicity test

Cytotoxicity test was done using MTT assay. The individual sealer discs were then added to the 96 well plates along with DMEM and in the negative control no sealer was added and incubated for 24 hrs, 72 hrs and 7 days.

After MTT assay, the viable cells in each well were calculated relative to control cells set to 100% and the absorbance was checked and recorded with 570nm using an ELISA microplate reader.

Cytotoxicity was assessed according to the percentage viability of fibroblast cells. The percentage viability was calculated relative to the control group.

- Non-cytotoxic with cell viability of more than 90%,
- Slightly cytotoxic with cell viability of 60 – 90%,
- Moderately cytotoxic with cell viability of 30 – 59% and
- Strongly cytotoxic with cell viability of less than 30%.

Results –

Statistical analysis was done by using one way ANOVA and Tukeys multiple posthoc procedures.

The results showed that Group1 AH Plus sealer (Epoxy resin based) was proved to be the most cytotoxic at all three time intervals of 24 hrs, 72 hrs and 7 days among the three sealers. Group 1 AH Plus sealer showed moderate cytotoxicity at all three intervals and the cytotoxicity of the sealer reduced from 24 hrs to 7^{th} day. The Group 2 GuttaFlow bioseal sealer (Silicone based) showed slight to no cytotoxicity over the three time intervals and showed the least cytotoxicity as compared to the other two sealers. Group 2 GuttaFlow bioseal sealer showed less cytotoxicity than Group 3 Bio-C sealer (Bioceramic based) however the difference in the cytotoxicity was not statistically significant at all three intervals of time.

Conclusion –

Group 2 GuttaFlow bioseal sealer (Silicone based) showed least cytotoxicity among the three sealers while Group 1 AH Plus (Epoxy resin based) showed the highest cytotoxicity.

Keywords – MTT assay; GuttaFlow bioseal; PDL Fibroblasts; Bio-C sealer; AH Plus

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INTRODUCTION

The main objectives of root canal treatment are elimination of bacteria and their by-products, complete disinfection and chemo-mechanical preparation of the root canals followed by a three-dimensional filling of the root canal using obturating material and appropriate sealer that will promote periapical healing and repair.[1,2]

The root canal sealer plays an important role in sealing of the dentinal tubules, preventing re-infection of the canals and also in promoting periradicular repair.[3] One of the important factor that affects periapical healing is the biocompatibility of the root canal sealer.[2,3,4] The root canal sealer may get accidentally extruded into the periapical area and come in direct contact with the periapical tissues leading to cytotoxicity, periapical inflammatory reaction or hinderance in periradicular healing and repair.[1,3,4,5]

Therefore, it is important to assess the biocompatibility of the sealers in an in-vitro set-up using various cell-based assays such as Tetrazolium Reduction Assays using compounds like MTT, MTS, XTT etc, Resazurin Reduction Assay, Protease Viability Marker Assay and Real-Time Assay for Viable Cells, before its clinical use to minimise their adverse toxic effects.[1,2,6,7] Among the cell-based assay used for determining cytotoxicity the Tetrazolium Reduction Assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction reagent is most widely adopted and remains popular in academic labs.[7]

Various cell lines used for in-vitro assessment of cytotoxicity include human periodontal ligament fibroblasts, gingival fibroblasts, L929 murine fibroblasts, primary human osteoblasts etc.[2]

AH Plus (Dentsply, Detrey, Konstanz, Germany) is an Epoxy resin based sealer widely used in clinical practice. It exhibits low setting shrinkage, good dimensional stability and is considered gold standard to be used as a comparison for various physiochemical properties with others sealers.[3,8,9] However, AH Plus sealer was reported to be cytotoxic due to minimal release of formaldehyde and also lacked bioactive potential.[3,9]

AH Plus is available in two pastes form. The composition of those are:- Paste A: Epoxy Resins, Calcium Tungstate, Zirconium Oxide, Silica, Iron Oxide Pigments, Aerosil; Paste B: Adamantane amine, N, N-Dibenzyl-5-oxanonane, TCD-Diamine, Calcium Tungstate, Zirconium Oxide, Aerosil.[2]

A novel formation of polydimethylsiloxane with gutta-percha powder combined with calcium silicate particles was launched in late 2015, named GuttaFlow bioseal sealer.[1] This Silicone based GuttaFlow bioseal sealer (Coltène/Whaledent AG, Altstatten, Switzerland) is a mixture of gutta-percha powder, zinc oxide, polydimethylsiloxane, a platinum catalyst, zirconium dioxide, bioactive glass and micro silver. It shows the ability for tissue regeneration and healing.[3]

Incorporation of calcium silicate particles allow the sealer to be used even in fluid contaminated environments and shows the ability to release calcium ions which facilitate nucleation of apatite deposits.[10,11] According to the recent literature, GuttaFlow bioseal has also been proposed as a root canal obturating material due to its properties of good flow and expansion on setting.[12,13] Furthermore, studies have proven that GuttaFlow bioseal sealer has good pushout bond strength due to the formation of interphase apatite deposits leading to its bond with dentin. The alkalizing property has shown to have stimulating effect on its sealing ability. This enhanced

sealing ability is due to formation of superficial calcium phosphate layer, resulting in reduction of voids. [14]

Recently, a novel premixed bioceramic sealer, Bio-C sealer is marketed in a single tube form.[15,16] The composition of this sealer is tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, zirconia oxide, silicone oxide, polyethylene glycol and iron oxide.[16,17]

Bio-C sealer possess the property of bioactivity which is attributed to release of Ca^{+2} ions and OH^- ions which not only aids in early mineralization but also imparts antibacterial properties by increasing the pH of the medium.[16,18,19]

Bio-C sealer, according to literature has shown to have good homogeneous dentinal tubule penetration and good filling ability due to its composition in nanometric scale. Furthermore, it has superior sealing ability as a result of formation of chemical bond with dentin due to hydroxyapatite formation.[20,21]

In previous studies, GuttaFlow bioseal sealer had been reported to possess lower cytotoxicity than AH26 sealer, MTA Fillapex sealer and Gutta Flow 2 sealer.[1,3,22,23,24] Bio-C sealer was also reported to be less cytotoxic as compared to TotalFill BC Sealer, Calcipex II sealer.'[15,16,19]

As there is no literature documented on cytotoxicity comparing the above three endodontic sealers; hence, the aim of this study was to evaluate and compare the cytotoxicity of Epoxy resin based (AH Plus sealer), Silicone based (GuttaFlow bioseal sealer) and Bioceramic based (Bio-C sealer) endodontic sealers using MTT assay at 24 hrs, 72 hrs and 7 days.

OBJECTIVES OF THE STUDY

AIM

To evaluate and compare the cytotoxic effect of the Epoxy resin based, Silicone based and Bioceramic based endodontic sealers on human periodontal ligament fibroblasts by MTT assay.

OBJECTIVES

1. To evaluate the cytotoxic effect of Epoxy resin based, Silicone based and Bioceramic based endodontic sealers on human periodontal ligament fibroblasts at 24 hrs, 72 hrs and 7 days by MTT assay.
2. To compare the cytotoxic effect of Epoxy resin based, Silicone based and Bioceramic based endodontic sealers on human periodontal ligament fibroblasts at 24 hrs, 72 hrs and 7 days by MTT assay.

HYPOTHESIS

NULL HYPOTHESIS: -

The Epoxy resin based, Silicone based and Bioceramic based endodontic sealers will not have any cytotoxic effect on the human periodontal ligament fibroblasts at 24 hrs, 72 hrs and 7 days.

ALTERNATE HYPOTHESIS: -

The Epoxy resin based, Silicone based and Bioceramic based endodontic sealers will have a cytotoxic effect on the human periodontal ligament fibroblasts at 24 hrs ,72 hrs and 7 days.

REVIEW OF LITERATURE

1. A study was done by Collado-González M et al, to evaluate the cytotoxic effects of GuttaFlow bioseal, GuttaFlow 2, MTA Fillapex and AH Plus endodontic sealers on human periodontal ligament stem cells. Samples were made by fabrication of disks of 5 x 2 mm dimension of each sealer. To evaluate cell viability, the cells were then exposed and incubated with these sealer discs for 24 hrs, 72 hrs and 168 hrs. Cytotoxicity was evaluated using MTT assay at each time interval. Also in this study, chemical composition of each was determined by energy-dispersive x-ray and eluates were analyzed by inductively coupled plasma mass spectrometry. The study concluded that, Gutta Flow bioseal and Guttaflow 2 showed highest cell viability and least cytotoxicity at all three intervals as compared to MTA Fillapex and AH Plus sealer.[1]

2. A study was done by Jagtap et al, to evaluate the cytotoxic effects of MTA Fillapex, Angelus, Apexit Plus, AH Plus and Tubli Seal endodontic sealers on human periodontal ligament fibroblast cells. Samples were made by fabrication of disks of 3 x 2 mm dimension of each sealer. Elutes of each sealer discs were made by placement of the specimens in media for 1hour, 7 days and 14 days intervals. Cytotoxicity was determined after each elution period after the elutes were incubated for 24 hours, using MTT assay and Trypan Blue staining. The study concluded that, AH Plus sealer showed least cytotoxicity while MTA Fillapex sealer showed highest cytotoxicity in both the techniques.[2]

3. A study was done by Saygili et al, to evaluate the cytotoxic effects of GuttaFlow Bioseal, GuttaFlow 2, AH-Plus and MTA Fillapex endodontic sealers on L929 murine fibroblasts. Samples discs were fabricated using teflon disks of 5 mm diameter and 3 mm thickness. Extracts of each sample were prepared and incubated at 3, 24, 72 and 168 hrs, cell viability at each time interval was assessed using MTT assay and Apoptosis was assessed using TUNEL assay. The study concluded that, GuttaFlow bioseal sealer showed least cytotoxicity and maximum cell viability at all intervals of time as compared to all other sealers.[3]

4. A study was done by Miletic' et al, to evaluate the cytotoxicity of AH plus sealer and RoekoSeal sealer on human cervical carcinoma (HeLa) cells and mouse skin fibroblasts (L929). The sealers were mixed and 0.02 ml increment of each sealer was added in 24-well plate, followed by addition of the cells and kept incubated for 1 hr, 24 hrs, 48 hrs, 7 days and 1 month after mixing. Samples from each material at different setting time was then collected and the cell viability was assessed by counting the number of cells under light microscope after addition of nigrosin dye. The study concluded that, AH Plus sealer showed significantly more cytotoxicity at 1 hr, 24 hrs and 48 hrs. However, the cytotoxicity reduced at day 7 and at 1 month.[4]

5. A study was done by Zhang et al, to evaluate the biocompatibility of iRoot SP root, AH Plus and ProRoot mineral trioxide aggregate (MTA) canal filling material on L929 mouse fibroblasts. Samples were fabricated in nonreactive plastic moulds of 5 x 2 mm dimensions of each sealer. Cytotoxicity assessment was done using direct contact using the Millipore filter diffusion test and MTT

assay. The study concluded that, AH Plus root canal sealer was significantly more cytotoxic to L-929 cells than other sealers.[5]

6. A study was done by Rodrigues et al, to evaluate and compare the cytotoxicity of Realseal, Apexit Plus, GuttaFlow and AH Plus endodontic sealers after setting at 24 hours, 7th day and 14th day using MTT assay on human gingival fibroblasts. In this study samples of the sealers were fabricated in sterile teflon moulds of dimensions 3 x 3 mm. Extracts of each sealer were prepared and cytotoxicity was evaluated using MTT assay and Spectophotometric analysis. The study concluded that, all the sealers showed varied amount of cytotoxicity which decreased from 24 hrs to 14^{th} day. Among the sealers GuttaFlow showed least cytotoxicity and Realseal sealer showed highest cytotoxicity.[8]

7. A study was done by Almeida et al, to evaluate physicochemical properties and cytotoxicity of AH Plus, MTA Fillapex and Total Fill BC endodontic sealer on 3T3 cells at 24 hrs, 48 hrs and 72 hours using MTT assay. Serial dilutions of the media conditioned with the sealers were performed in the concentrations of 50 mg/ mL, 10 mg/mL, 5 mg/mL, 1 mg/mL and 0 mg/mL (negative control) and exposed on to the cells. Cytotoxicity analysis was performed using MTT assay at each time interval of 24 hrs, 48 hrs and 72 hrs. Also, in this study physicochemical properties such as radiopacity, flow rate, setting time, release of Ca^{+2} ions, pH and volumetric changes were evaluated. The study concluded that, AH Plus sealer showed higher cytotoxicity than TotalFill BC Sealer, but showed lower initial and final setting time and lower volumetric changes.[9]

8. A study was done by Silva et al, to evaluate the biocompatibility and bioactive potential of Bio-C sealer, AH Plus sealer and Sealer Plus BC in subcutaneous tissue of rats. In this study, polyethylene tubes filled with materials and for control group, empty tubes were implanted in the subcutaneous tissues of rats. After 7, 15, 30 and 60 days, the tubes along with connective tissue were removed. Inflammatory cells (ICs)/mm2 and immunolabeled cells for interleukin (IL)-6 were evaluated. Also, Osteocalcin and von Kossa analysis were performed. The study concluded that, though Bio-C sealer induced mild inflammatory response, it is considered biocompatible as it favored repair and also contribute to the periapical tissue mineralization process due to its bioactive potential.[15]

9. A study was done by López-García et al, to investigate the cytocompatibility and mineralization potential of Bio-C sealer, TotalFill BC Sealer and AH Plus on human periodontal ligament stem cells (hPDLSCs) using MTT assay and Alizarin Red assay. Also, cell migration, cell morphology, Cell Attachment and Surface Morphology were analyzed in this study, using scanning electron microscopy (SEM) and Energy Dispersive X-ray microanalysis (EDX) respectively. The study concluded that, Bio-C sealer and TotalFill BC Sealer demonstrated better cytocompatibility in terms of cell viability, migration, cell morphology, cell attachment and mineralization capacity than AH Plus.[16]

10. A study was done by López-García et al, to evaluate the biocompatibility of two hydraulic materials, Bio-C sealer and Bio-C repair on periodontal ligament stem cells. The study evaluated metabolic activity, cell migration and cell survival using the MTT assay, wound-healing assays and Annexin assay respectively.

Analysis was done using Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX). The study concluded that, Bio-C Repair material displayed higher cell viability, cell adhesion and migration rates as compare to Bio-C sealer.[18]

11. A study was done by Okamura et al, evaluated the biocompatibility of Bio-C sealer and Calcipex-II sealer. The cytotoxicity of the sealers was analyzed using MTT assay on V-79 cell lines. Biocompatibility of the sealers with the periapical tissues was evaluated by performing endodontic treatment on teeth of beagle dogs using these sealers, followed by histological evaluation of the periapical tissues of the tooth on 28^{th} day and 90^{th} day. Also in this study, chemical elements of the materials were analyzed using scanning electron microscopy (SEM), X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR). The study concluded that, Bio-C sealer is biocompatible due to its lower cytotoxicity than Calcipex II sealer and is safe to be used in close proximity to the periapical tissues as no inflammatory response was seen and healing was favored.[19]

12. A study was done by Ferreira et al, evaluation of cytotoxic effects of GuttaFlow bioseal sealer and AH26 Epoxy Resin sealer was done using MTT assay and SRB assay on MDPC-23 odontoblast cell cultures. The study also evaluated cell cycle and cell-death types using cytometry and mineralization capacity using the Alizarin Red assay. Metabolic activity of the cells was evaluated at 24 hrs, 48 hrs, 72 hrs and at 120 hrs. The study concluded that, GuttaFlow bioseal showed higher biocompatibility as compared to AH 26 sealer.[22]

13. A study was done by Santos et al, to evaluate the biocompatibility of GuttaFlow Bioseal, GuttaFlow 2 and AH Plus endodontic sealers in rat subcutaneous tissue. Four subcutaneous tissue implants, made of mixed sealer in polyethylene tubes were placed in the rats. Biocompatibility was analyzed based on inflammatory scored graded according to the histological analysis of the subcutaneous tissue of the rats on day 8 and day 30. The study concluded that, all the sealers showed initial inflammatory response which reduced from day 8 to day 30. Among the sealers, GuttaFlow bioseal sealer showed minimum inflammatory response and showed maximum macrophage infiltration.[23]

14. A study was done by Rodríguez-Lozano et al, to evaluate the cell viability, cell migration and cell morphology on human periodontal ligament stem cells (hPDLSCs), after their exposure to GuttaFlow bioseal, GuttaFlow 2, MTA Fillapex and AH Plus sealers, cell attachment was evaluated by directly seeding the cells onto the material surfaces and analysis was done using scanning electron microscopy (SEM). Also, the study evaluated the effect of sealers on cementum protein1 (CEMP1), cementum-derived attachment protein (CAP), bone sialoprotein (BSP), ameloblastin (AMBN), amelogenin (AMELX) and alkaline phosphatase (ALP) gene expression on hPDLSCs using qPCR and immunofluorescence (IF). GuttaFlow bioseal and GuttaFlow 2 reported higher cell viability as compared to AH Plus and MTA Fillapex at 72 hrs interval and showed higher level of AMELX, AMBN, CEMP1 and CAP expression than the control.[24]

15. A study was done by Zoufan et al, to evaluate cytotoxicity of GuttaFlow, Endosequence BC, Tubli-seal and AH Plus endodontic sealers on L929 mouse

fibroblast cells at time intervals of 24 hrs and 72 hrs. The cytotoxicity of the different sealers was evaluated in freshly mixed and set state. Twelve eluate groups were made for each sealer and the cytotoxicity was evaluated. The study concluded that, GuttaFlow and BC sealers showed lower cytotoxicity than AH Plus and Tubli-seal sealers.[25]

16. A study was done by Delfino et al, to evaluate immunoinflammatory response and bioactive potential of two endodontic sealers, GuttaFlow bioseal, MTA Fillapex and Endofill (EF) on rat subcutaneous tissue, for a period of 7, 15, 30 and 60 days. Polyethylene tubes with GuttaFlow bioseal, MTA Fillapex and Endofill were implanted into the subcutaneous tissue of rat. The study evaluated the immunoinflammatory response and bioactive potential by checking the capsule thickness, inflammatory reaction, interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), caspase-3, TUNEL-positive cells, von Kossa and ultrastructural features. The study concluded that, the immunoinflammatory response due to GuttaFlow bioseal sealer was less compare to MTA Fillapex and Endofill sealer.[26]

17. A study was done by Zhou et al, to evaluate the cytotoxicity of two calcium silicate–containing endodontic sealers EndoSequence BC, MTA Fillapex and AH Plus on human gingival fibroblasts using flow cytometry and the adhesion of the fibroblasts to the surface of the set materials using scanning electron microscopy. Extracts from each specimen were made from both fresh and set materials for 4 consecutive weeks. The collected extracts were diluted to 1:2, 1:8, 1:32, and 1:128 with DMEM to achieve 4 concentrations of each extract. The cells were then exposed to these extracts from fresh and set materials. The study concluded that,

AH Plus was cytotoxic only for extracts from freshly mixed sealer. However, it was more cytotoxic as compared to EndoSequence BC sealer.[27]

18. A study was done by Karapınar-Kazandag et al, to evaluate the cytotoxicity of AH Plus, RoekoSeal, EndoREZ, Epiphany and Active GP endodontic sealers on L929 mouse fibroblast cells and primary human dental pulp cells using MTT Assay. Extraction of specimens was performed after setting in cell growth medium for 1, 4 and 7 days. These specimens were used in undiluted, 50% and 25% diluted eluates. These eluates were exposed to the cultured cells for 24 hrs and 72 hrs, cytotoxicity was analyzed. The study concluded that, low to no cytotoxicity was observed with RoekoSeal sealer, AH Plus sealer and EndoREZ sealer. All the sealers exhibited varying degrees of cytotoxicity.[28]

19. A study was done by Dhopavkar et al, in which cytotoxicity and genotoxicity of three endodontic sealer were evaluated using MTT assay and Comet Assay respectively, on human periodontal ligament fibroblast cells. Discs for each sealer of 2 x 2 mm were fabricated on teflon moulds and allowed to set for 24 hrs, following which the cells were exposed to these sealer discs in 96 well plate and incubated for 24 hrs and 48 hrs. Cytotoxicity was evaluated using MTT assay at both the time intervals, also genotoxicity of the sealers was evaluated using Comet Assay. The study concluded that, AH Plus sealer showed lower cytotoxicity and genotoxicity as compared to MTA Fillapex sealer and showed higher cytotoxicity and genotoxicity as compared to GuttaFlow 2 sealer.[29]

20. A study was done by Deog-Gyu Seo et al, to evaluate the biocompatibility and mineralization Activity of EndoSequence BC Sealer, BioRoot RCS, Endoseal MTA and AH Plus. The experimental discs of dimension 6 x 3 mm were made and allowed to set for 72 hrs, following which cytotoxicity was evaluated using MTT assay. Also, the study evaluated cell migration and cell morphology, by Scratch wound healing method and Scanning Electron Microscopy. Also, mineralization ability was assessed using Alizarin red staining assay. The study concluded that, AH Plus sealer showed higher cytotoxicity compared to other sealers.[30]

MATERIALS AND METHODS

SOURCE OF DATA:

This in-vitro study was conducted in the Department of Conservative Dentistry and Endodontics, KLE V.K Institute of Dental Sciences, KLE Academy of Higher Education & Research (KLE University), Belagavi.

For this study, freshly extracted human maxillary and mandibular premolar teeth with intact periodontal ligament indicated for orthodontic extraction were collected from Department of Oral and Maxillofacial Surgery, KLE V.K Institute of Dental Sciences, KLE Academy of Higher Education & Research (KLE University), Belagavi.

The laboratory procedure for cytotoxicity analysis using MTT assay was carried out at Dr Prabhakar Kore's Basic Sciences Research Centre (BSRC), Belagavi.

INCLUSION CRITERIA

- Human maxillary and mandibular premolar teeth indicated for orthodontic extraction.

EXCLUSION CRITERIA: -

- Teeth with visible caries, external resorption seen radiographically and showing signs clinically of inflamed periodontal tissue before extraction.

MATERIALS USED FOR THE STUDY:

1. Human periodontal ligament fibroblasts
2. Dulbecco's Modified Eagle Medium (DMEM)
3. 10% Fetal bovine serum
4. MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
5. Dimethyl sulfoxide solvent (DMSO)
6. AH Plus (Epoxy resin based) (Dentsply DeTrey)
7. GuttaFlow bioseal (Silicone based) (Coltene/ Whaledent)
8. Bio-C sealer (Bioceramic sealer) (Angelus, Londrina, PR, Brazil)

ARMAMENTARIUM USED FOR THE STUDY

1. 96 well plate
2. Mixing pad
3. Spatula
4. Micropipette
5. Micropipette tips (0-200 µl)
6. CO_2 incubator (Brunswick Eppendorf)
7. ELISA microplate reader (LISA Plus; 570nm)

SAMPLE SIZE ESTIMATION:

$S = \frac{S_1 + S_2}{2}$

Z_α= 2.58 at 1% α error.

Z_β= 1.682 at 5% β error.

S1=84.44

S2=200

d= 202.32

$$n = \frac{2\sigma^2 (Z_\alpha + Z_\beta)^2}{d^2} = 18 \text{ (in each group)}$$

Sample size will be 18 per group including the control group.

METHODOLOGY

- Cell culture of human periodontal ligament fibroblasts

Maxillary or mandibular premolar teeth indicated for orthodontic extraction were extracted as atraumatically as possible by oral surgeon, were procured and handled according to OSHA guidelines and were placed in phosphate buffer saline solution. The excised PDL tissues were placed in tissue culture dishes, following which establishment of explant cultures was done.

The subsequent sub-cultures were prepared in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 1000 units/ml penicillin 10 mg/ml streptomycin. Confluent cells after incubation were detached using 0.25% trypsin.

- Preparation of test materials

Test groups made were: -

Group 1: - AH Plus (Epoxy resin based sealer)

Group 2: - GuttaFlow bioseal (Silicone based sealer)

Group 3: - Bio-C sealer (Bioceramic based sealer)

Group 4: - Negative control (No sealer)

The endodontic sealers were mixed according to the manufacturer's instructions under sterile aseptic conditions and made into eighteen separate discs of each sealer using cylindrical teflon moulds (2mm diameter and 2mm in length). The test samples were allowed to set in a humid chamber at 37 ℃ for 24 hrs. All the discs were sterilized by keeping them in laminar air flow for 15-20 minutes.

- Cytotoxicity test

Cytotoxicity test was done by MTT assay. The cell lines were cultured in DMEM medium which was supplemented with 10% Fetal bovine serum (FBS) and 1% antibiotic and antimycotic solution. Following which seeding of the cells was done at the density of 5000-10000 cells\well in a 96 well flat bottom microplate and maintained overnight at 37℃ in 95% humidity and 5% CO_2.

The individual sealer discs were then added to the 96 well plates along with DMEM and in negative control where no sealer disc was added.

The cells were then incubated for another 24 hrs, 72 hrs, and 7 days. After incubation, the discs were removed and the cells in the well plates were washed twice in phosphate buffer solution and 20µl of 0.5% MTT reagent was added to each well, after which it was incubated at 37°C for 4 hrs.

After 4 hrs, 100µl of Dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. Following which, viable cells in each well were calculated relative to control cells set to 100% using the absorbance that was checked and recorded at 570nm wavelength using an ELISA microplate reader.

- Percentage of cell viability was calculated using the formula[8]

$$\% \text{ Of cell viability} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Cytotoxicity was rated based on cell viability relative to control as given by[8, 31]:

- Non cytotoxic (> 90% cell viability),
- Slightly cytotoxic (60 – 90% cell viability),
- Moderately cytotoxic (30 – 59% cell viability),
- Strongly cytotoxic (< 30% cell viability).

The percentage of cell viability for each sealer was recorded and the results were tabulated and subjected to statistical analysis.

FLOWCHART DEPICTING THE STUDY DESIGN

- **CELL CULTIVATION**

Premolar teeth indicated for orthodontic extraction were extracted as atraumatically as possible by oral surgeon and were procured and handled according to OSHA guidelines and were placed in phosphate buffer saline solution.

↓

Periodontal ligament tissue from these freshly extracted non-infected teeth were excised and placed in tissue culture dishes

↓

Establishment of explant cultures

↓

Subsequent sub-culturing of the periodontal ligament fibroblast cells was done in DMEM containing 10% Fetal bovine serum +1000 units/ml penicillin +10 mg/ml streptomycin.

↓

Confluent cells were detached using 0.25% trypsin

↓

Cell seedings were then placed in respective wells in 96 well plate.

- **TEST SAMPLE PREPARATION**

The endodontic sealers were mixed according to the manufacturer's instructions

↓

Test groups

↓

GROUP I: - AH Plus

GROUP II: - GuttaFlow bioseal

GROUP III: - Bio-C sealer

GROUP IV: - Control group (No sealer)

↓

Eighteen sealer discs were made using sterile teflon moulds of 2 × 2mm

↓

The materials were allowed to set for 24 hrs in humid chamber at 37℃

- **CYTOTOXICITY TESTS**

Seeding of the cells was done in respective wells in 96 well plates at a density of 5000-10,000 cells per well

↓

The cells were maintained at 37℃ in 95% humidity and 5 % CO_2 for 24 hrs.

↓

Test compounds discs were then added into the wells containing the PDL fibroblast cells along with DMEM

↓

In the negative control no sealer disc was added.

↓

The materials were tested at 24 hrs, 72 hrs, and 7 days

↓

After incubation, the discs were then removed, the cells were washed with phosphate buffer solution

↓

20µl of 5% of MTT reagent was then added and incubated for 4 hrs

↓

100µl of Dimethyl sulfoxide was then added to dissolve the formazan crystals

↓

Viable cells in each well were calculated relative to control cells set to 100% using the absorbance that was checked and recorded at 570nm wavelength using an ELISA microplate reader.

- **PERCENTAGE OF CELL VIABILITY WILL BE CALCULATED USING THE FORMULA** [8]

$$\% \text{ Of cell viability} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Cytotoxicity was rated based on cell viability relative to control as given by[8, 31]:

- Non cytotoxic (> 90% cell viability),
- Slightly cytotoxic (60 – 90% cell viability),
- Moderately cytotoxic (30 – 59% cell viability),
- Strongly cytotoxic (< 30% cell viability).

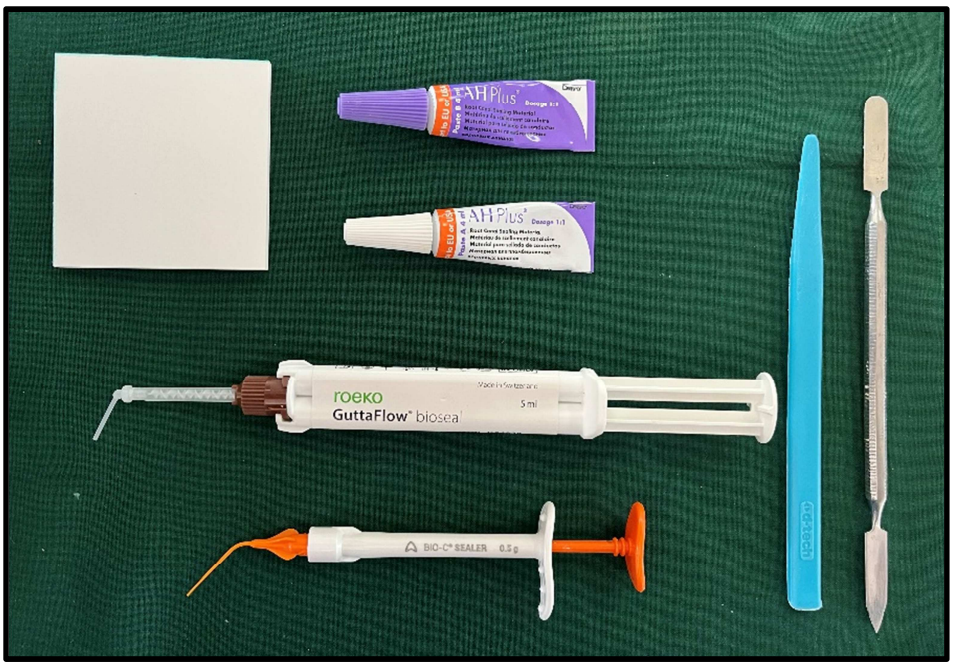


Fig. no. 1– Test materials - root canal sealers

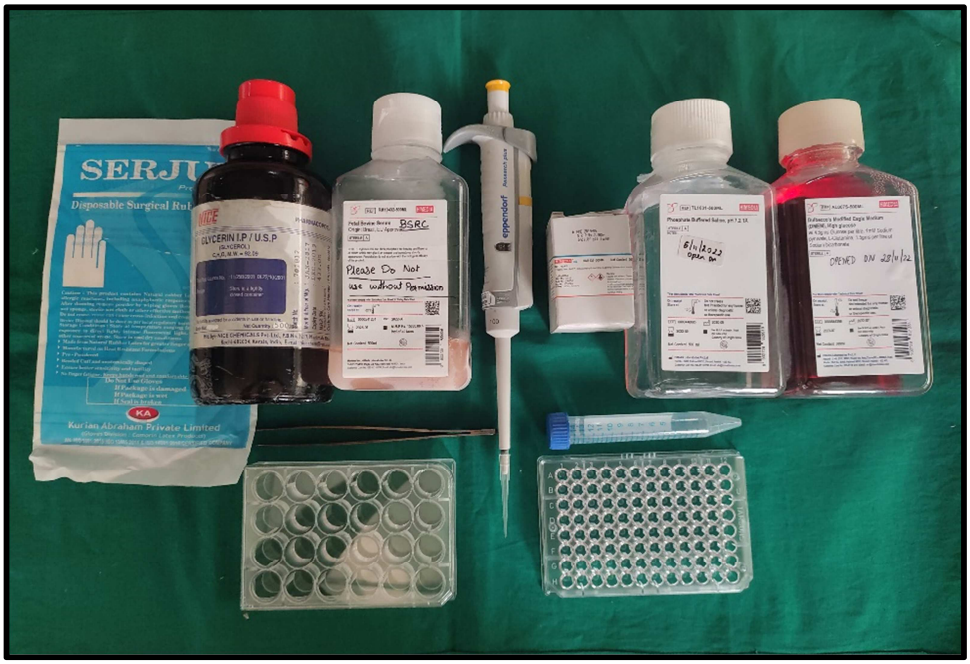


Fig. no. 2 - Armamentarium for Cytotoxicity

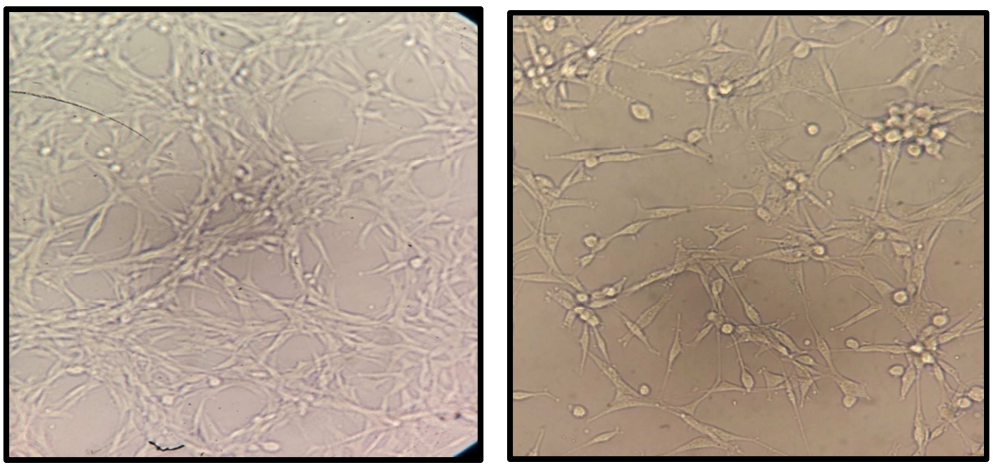


Fig. no. 3 - Human periodontal Ligament Fibroblasts

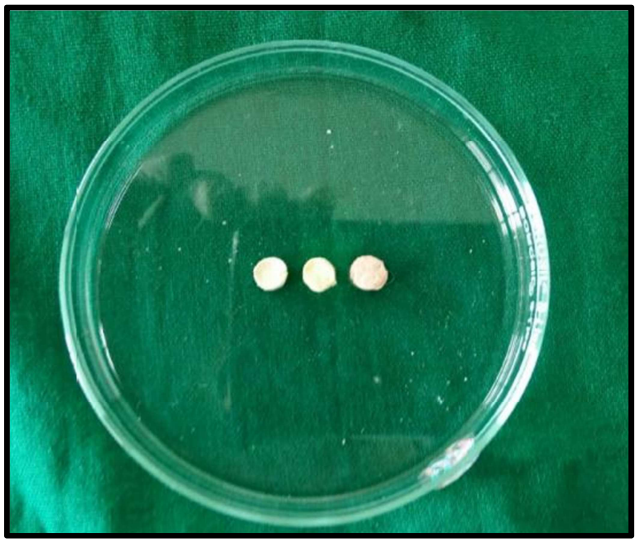


Fig. no 4 - Sealer discs prepared from teflon moulds

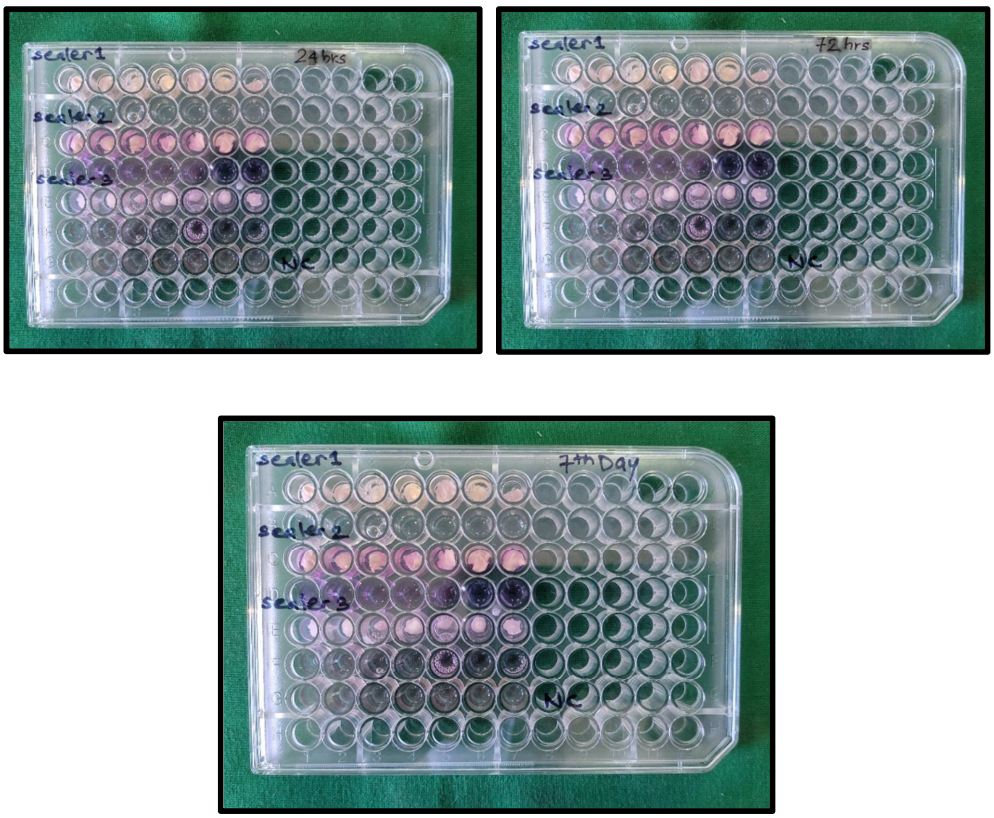


Fig. no. 5 - 96 Well plate with test compound for 24 hrs, 72 hrs and 7 days.

Fig. no. 6 - CO_2 Incubator

Fig. no. 7 - ELISA Spectrophotometer Reader

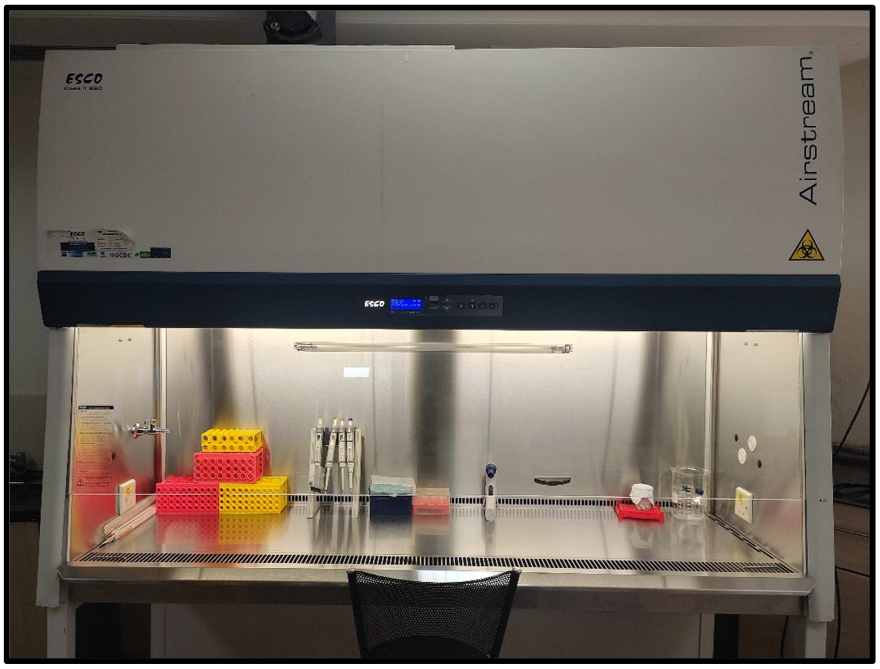


Fig. no. 8 - Laminar air flow

RESULTS

Table no 1A: - Master chart showing optical density values; mean of optical density and percentage of cell viability at 24 hrs.

Cytotoxicity of test compounds at 24 hrs.				
Serial number	**Compound name**	**Optical density**	**Mean of optical density**	**Cell viability**
1.	**Group 1-AH Plus**	0.112 0.112 0.108 0.108 0.105	0.118	42.0%
		0.169		
2	**Group 2- GuttaFlow bioseal**	0.249 0.265 0.265 0.193 0.209	0.238	84%
		0.255		
3	**Group 3- Bio-C sealer**	0.173 0.172 0.226 0.270 0.162	0.197	69.5%
		0.192		
4	**Negative Control**	0.308 0.317 0.254 0.236 0.241 0.303	0.354	100%

Table no.1B: - Master chart showing optical density values; mean of optical density and percentage of cell viability at 72 hrs

Cytotoxicity of test compounds at 72 hrs				
Serial number	**Compound name**	**Optical density**	**Mean of optical density**	**Cell viability**
1.	**Group 1-AH Plus**	0.133 0.2 0.196 0.203 0.182	0.184286	44.5%
		0.199		
2	**Group 2- GuttaFlow bioseal**	0.305 0.551 0.463 0.469	0.392143	94.89%
		0.308		
		0.292		
3	**Group 3- Bio-C sealer**	0.341 0.213 0.351 0.354	0.327714	79.42%
		0.355		
		0.353		
4	**Negative Control**	0.425 0.288 0.521 0.475 0.402 0.372	0.413143	100%

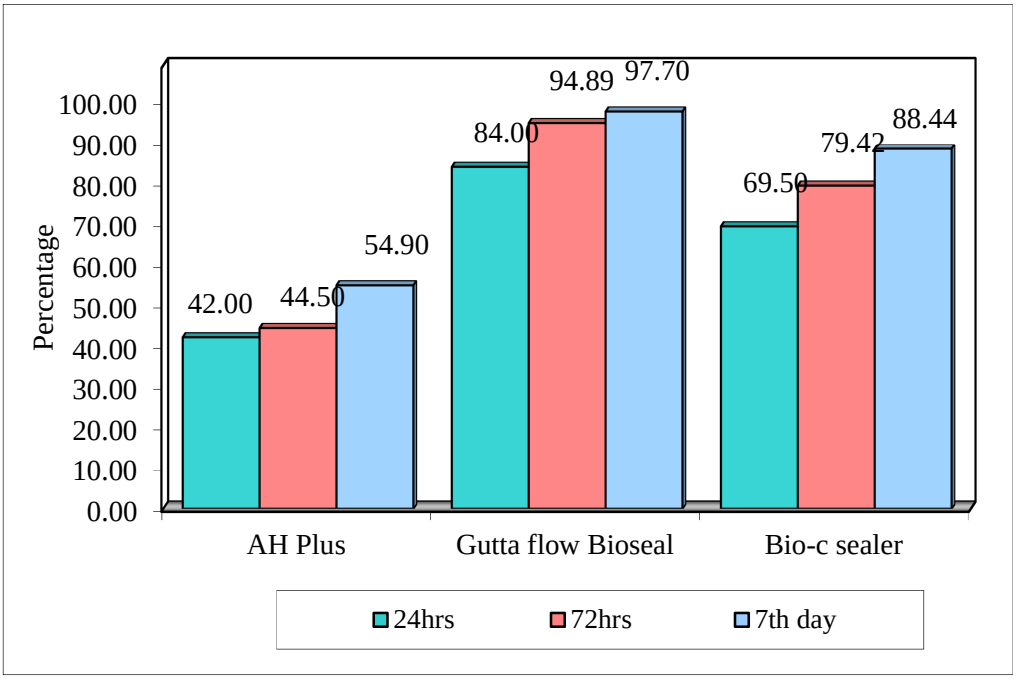
Table no 1C: - Master chart showing optical density values; mean of optical density and percentage of cell viability at 7th day

Cytotoxicity of test compounds at 7th day				
Serial number	**Compound name**	**Optical density**	**Mean of optical density**	**Cell viability**
1.	**Group 1-AH Plus**	0.228 0.226 0.339 0.234	0.2714	54.9%
		0.338		
		0.264		
2	**Group 2- GuttaFlow bioseal**	0.585 0.488 0.473 0.406	0.483	97.7%
		0.421		
		0.527		
3	**Group 3- Bio-C sealer**	0.391 0.373 0.321 0.494	0.437	88.44%
		0.512		
		0.497		
4	**Negative Control**	0.537 0.385 0.408 0.511 0.525 0.637	0.4941	100%

Table no 2: - Summary of percentage cell viability of three different sealer groups at 24 hrs, 72 hrs and 7th day

Groups	24 hrs (in %)	72 hrs (in %)	7th day (in %)
AH Plus	42.0	44.5	54.9
GuttaFlow bioseal	84.0	94.9	97.7
Bio-C sealer	69.5	79.4	88.4

Graph no 1: Percentage of cell viability changes from 24 hrs to 72 hrs and day 7 in three sealer groups



Observation from table no 1A, 1B, 1C and 2: -

The tables depict optical density values; mean of optical density and percentage of cell viability of the three sealers relative to the negative control at 24 hrs 72 hrs and 7^{th} day.

At 24 hrs, AH Plus sealer showed percentage of viable cells 42.0% which increased to 44.5% at 72 hrs and increased to 54.9% on the 7^{th} day.

At 24 hrs GuttaFlow bioseal sealer showed percentage of viable cells 84.0% which increased to 94.89% at 72 hrs and increased to 97.7% on the 7^{th} day.

At 24 hrs Bio-C sealer showed percentage of viable cells 69.5% which increased to 79.42% at 72 hrs and increased to 88.44% on the 7^{th} day.

Table no 2 depicts summary of percentage cell viability of the three endodontic sealers at three time intervals i.e., at 24 hrs, 72 hrs and 7 days.

Graph 1 depicts the cytotoxicity changes from 24 hrs to day 7, according to the percentage of cell viability.

Table 3: Comparison of four groups using optical density at different time intervals by one way ANOVA

Treatment time points	AH Plus		GuttaFlow bioseal		Bio-C sealer		Negative Control		F-value	p-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
24 hrs	0.1190	0.0246	0.2393	0.0307	0.1992	0.0415	0.2765	0.0367	23.6810	0.0001*
72 hrs	0.1855	0.0267	0.3980	0.1101	0.3278	0.0565	0.4138	0.0813	11.4882	0.0001*
Day 7	0.2715	0.0537	0.4833	0.0667	0.4313	0.0799	0.5005	0.0923	11.7526	0.0001*
Changes from										
24 hrs to 72 hrs	0.0665	0.0324	0.1587	0.1099	0.1287	0.0573	0.1373	0.1100	1.3192	0.2960
24 hrs to Day 7	0.1525	0.0624	0.2440	0.0509	0.2322	0.0886	0.2240	0.0975	1.7140	0.1963
72 hrs to Day 7	0.0860	0.0553	0.0853	0.1488	0.1035	0.0771	0.0867	0.1235	0.0397	0.9891

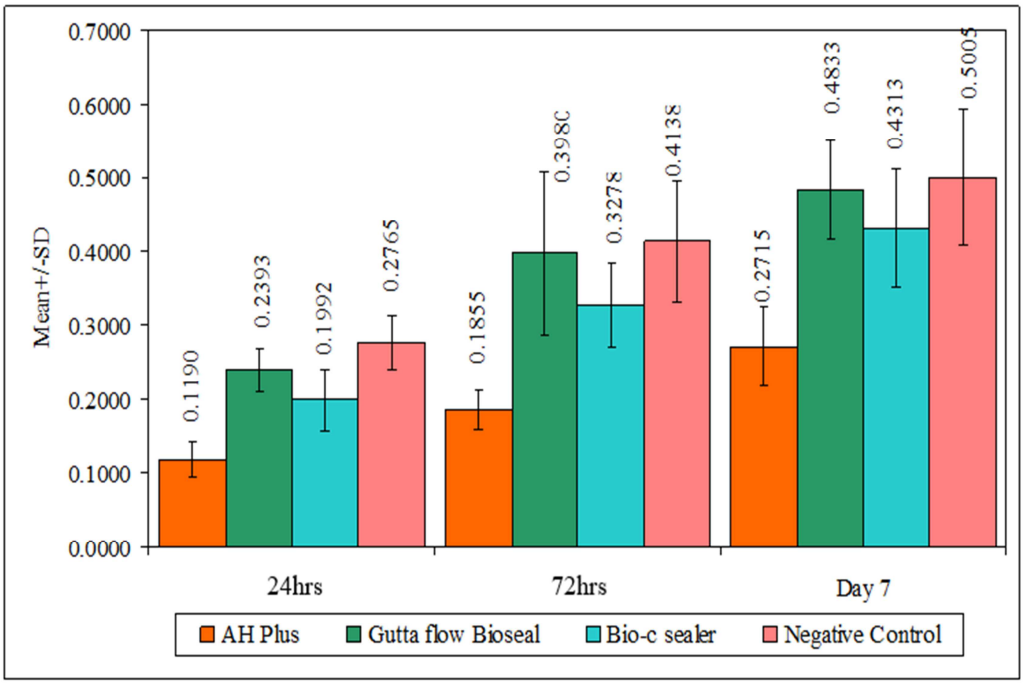
*$p<0.05$ indicates significant

Table no 4: Comparison of four groups at different time intervals using optical density by repeated measures of ANOVA

Groups	24 hrs		72 hrs		Day 7		Repeated ANOVA		
	Mean	SD	Mean	SD	Mean	SD	F-value	p-value	Effect size
AH Plus	0.1190	0.0246	0.1855	0.0267	0.2715	0.0537	26.9990	0.0001*	0.8400
GuttaFlow bioseal	0.2393	0.0307	0.3980	0.1101	0.4833	0.0667	14.9870	0.0010*	0.7500
Bio-C sealer	0.1992	0.0415	0.3278	0.0565	0.4313	0.0799	28.5200	0.0001*	0.8510
Negative Control	0.2765	0.0367	0.4138	0.0813	0.5005	0.0923	12.4580	0.0020*	0.7180

*$p<0.05$ indicates significant

Graph no 2: Comparison of the four groups with respect to optical densities at 24 hrs, 72 hrs and 7th day



Observation from table no. 3 and 4 and graph 2: -

Table no. 3 and Table no. 4 depicts comparison of four groups using optical density at different treatment time intervals by one way ANOVA and repeated measures of ANOVA respectively. Difference in standard deviation of optical densities at all three intervals i.e., at 24 hrs, 72 hrs and 7th day is compared for each sealer group.

The tables depict that, the difference in optical densities at 24 hrs, 72hrs and 7 days for all the sealer groups was statistically significant.

Graph 2 depicts comparison of the three sealer groups and the negative control group with respect to optical densities at 24 hrs, 72 hrs and 7th day

Table 5: Pair wise comparison of four groups with optical density at different time intervals by Tukeys multiple posthoc procedures

Time points	Pair wise comparison of four groups					
	AH Plus vs GuttaFlow bioseal	AH Plus vs Bio-C sealer	AH Plus vs Negative Control	GuttaFlow bioseal vs Bio-C sealer	GuttaFlow bioseal vs Negative Control	Bio-C sealer vs Negative Control
24 hrs	p=0.0002*	p=0.0031*	p=0.0002*	p=0.2046	p=0.2619	p=0.0042*
72 hrs	p=0.0006*	p=0.0183*	p=0.0004*	p=0.3932	p=0.9830	p=0.2288
7^{th} day	p=0.0006*	p=0.0070*	p=0.0003*	p=0.6291	p=0.9780	p=0.3975
	Changes from					
24 hrs to 72 hrs	p=0.2634	p=0.5885	p=0.4829	p=0.9260	p=0.9713	p=0.9980
24 hrs to 72 hrs	p=0.2029	p=0.3084	p=0.3991	p=0.9933	p=0.9692	p=0.9978
72 hrs to Day 7	p=1.0000	p=0.9921	p=1.0000	p=0.9911	p=1.0000	p=0.9929

*$p<0.05$ indicates significant

Observation from table no 5: -

The table no. 5 depicts pair wise comparison of the four groups at 24 hrs, 72 hrs and 7^{th} day using Tukeys multiple posthoc procedures

At 24 hrs, there was statistically significant difference in the optical densities between Group 1 AH Plus sealer, Group 2 GuttaFlow bioseal sealer and Group 3 Bio-C sealer. Also, there was statically significant difference in optical densities between the negative control, Group 1 AH Plus sealer and Group 3 Bio-C sealer.

However, difference between the optical densities between Group 2 GuttaFlow bioseal sealer and Group 3 Bio-C sealer and between Group 2 GuttaFlow bioseal and Negative control showed no statically significant difference.

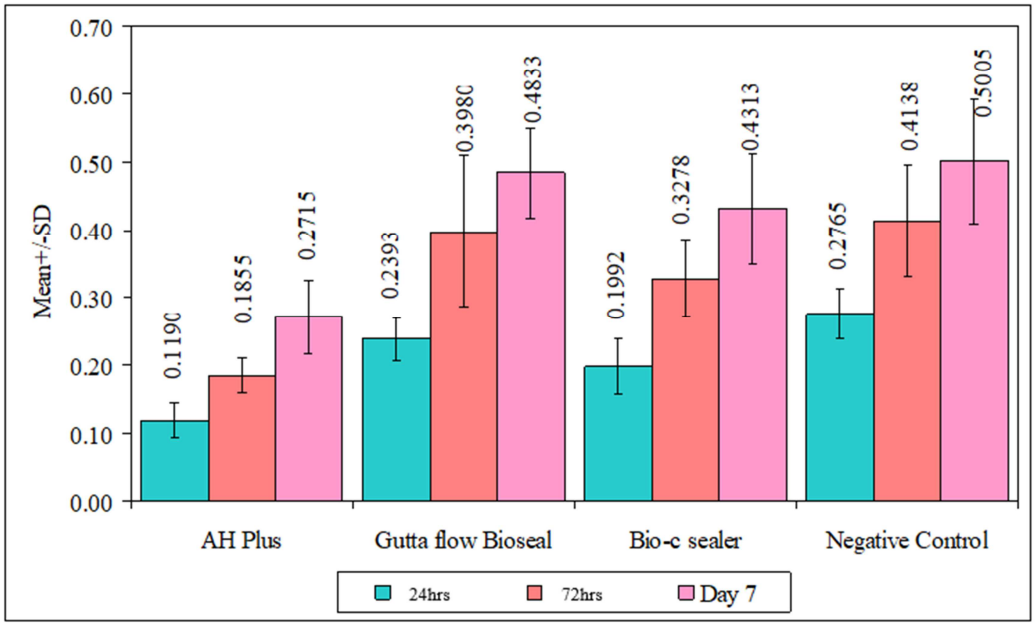
At 24 hrs and at day 7, the difference in the mean optical densities was statistically significant between the groups Group 1 AH Plus vs Group 2 GuttaFlow bioseal, Group 1 AH Plus vs Group 3 Bio-C sealer and Group 1 AH Plus vs Negative Control. While the difference was not significant between Group 2 GuttaFlow bioseal and Group 3 Bio-C sealer, Group 2 GuttaFlow bioseal and Negative Control and Group 3 Bio-C sealer vs Negative Control.

Table no 6: Comparison of four groups with change in optical density at different time intervals by dependent t-test

Groups	24 hrs to 72 hrs				24 hrs to Day 7				72 hrs to Day 7			
	Mean Diff.	SD Diff.	t-value	p-value	Mean Diff.	SD Diff.	t-value	p-value	Mean Diff.	SD Diff.	t-value	p-value
AH Plus	-0.0665	0.0324	-5.0270	0.0040*	-0.1525	0.0624	-5.9870	0.0020*	-0.0860	0.0553	-3.8090	0.0130*
GuttaFlow bioseal	-0.1587	0.1099	-3.5350	0.0170*	-0.2440	0.0509	-11.735	0.0001*	-0.0853	0.1488	-1.4050	0.2190
Bio-C sealer	-0.1287	0.0573	-5.4990	0.0030*	-0.2322	0.0886	-6.4190	0.0010*	-0.1035	0.0771	-3.2890	0.0220*
Negative Control	-0.1373	0.1100	-3.0590	0.0280*	-0.2240	0.0975	-5.6260	0.0020*	-0.0867	0.1235	-1.7180	0.1460

*p<0.05 indicates significant

Graph no 3: Comparison of the four groups with respect to changes in optical densities at 24 hr, 72 hrs and 7th day



Observation from table no 6

The table 6 depicts difference in optical densities in individual groups from 24 hrs to 72 hrs and 24 hrs to 7th day and 72 hrs to 7th day.

The Group 1 AH Plus showed the difference in optical densities to be statistically significant from 24 hrs to 72 hrs, 72 hrs to 7th day and from 24 hrs to 7th day.

In Group 2 GuttaFlow bioseal the difference in optical densities was statistically significant from 24 hrs to 72 hrs and from 24 hrs to day 7, but not from 72 hrs to day 7.

In Group 3 Bio-C sealer the difference in optical densities was statistically significant from 24 hrs to 72 hrs, and from 24 hrs to day 7 and from 72 hrs to day 7.

DISCUSSION

Endodontic sealers come directly in contact with the periapical tissues and may cause cellular damage thereby leading to delayed/no periapical healing further causing failure of the endodontic treatment[27]. Therefore, cellular toxicity and bioactivity of the sealers is mandatory to avoid detrimental effects on living cells.[27,32,33] Hence, the purpose of this study was to assess and evaluate the toxic effects of the sealers on human periodontal ligament fibroblast cells.

In this study, the cytotoxicity of three sealers was evaluated. The three sealers included in the study were; Group 1: AH Plus (Epoxy resin based), Group 2; GuttaFlow bioseal (Silicone based) Group 3: Bio-C sealer (Bioceramic based)

A variety of techniques are available for evaluation of biocompatibility of materials among which cell viability assays are most commonly employed.[34] In this study, MTT assay was used to assess the cytotoxicity of the sealers. MTT assay i.e., 3, (4, 5- dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide assay has various advantages, such as ease of use, good reproducibility and is widely adopted assay according to the literature.[3,33,34]

In MTT assay, the MTT reagent (tetrazolium salt) is metabolized in mitochondria of the viable cells to form insoluble formazan crystals, which on addition of a solvent, like dimethyl sulphoxide (DMSO) imparts a specific colour. This change in colour is spectrophotometrically analysed using ELISA microplate reader at 570 nm wavelength.[3,31,34]

According to literature, in-vitro cytotoxicity analysis should be performed on cell lines derived from human tissue which is of utmost concern regarding the material to be used.[35,36] In case of accidental extrusion of the sealer, there is direct contact of the sealer with the PDL cells, of which fibroblasts form the most predominant type [1], therefore use of cultured human PDL fibroblasts for the study is justified. According to Huang et al, use of human fibroblasts would help to reduce the species concerning bias.[35]

In this study, cytotoxicity was assessed according to the percentage viability of fibroblast cells. The percentage viability was calculated relative to the control group as given by Rodrigues et al, which was used to analyse the cytotoxicity as follows: - non-cytotoxic with cell viability of more than 90%, slightly cytotoxic with cell viability of 60 – 90%, moderately cytotoxic with cell viability of 30 – 59% and strongly cytotoxic with cell viability of less than 30% .[8]

In this study, the null hypothesis i.e., the three sealers (Epoxy Resin based, Silicone based and Bioceramic based sealer) will not have any cytotoxic effect on human periodontal ligament fibroblasts was rejected. In this study, Group 1 AH Plus sealer has been proved to be the most cytotoxic at all three time intervals of 24 hrs, 72 hrs and 7 days among the three sealers.

Group 1 AH Plus sealer showed moderate cytotoxicity at all three intervals i.e., the cell viability at 24 hrs was 42% at 72 hrs was 44.5% and on 7th day interval was 54.9%. This moderate cytotoxicity can be attributed to the release of formaldehyde or due to epoxy resin content or amine content of the Group 1 AH Plus sealer, according to literature.[2,4,5,8,9,25,28]

Also, the cytotoxicity of the sealer reduced from 24 hrs to 7th day. These results are in accordance with the previous cytotoxicity study analysis of Group 1 AH Plus sealer. [25]

The Group 2 silicone-based GuttaFlow bioseal sealer showed slight to no cytotoxicity over the three time intervals and showed the least cytotoxicity as compared to the other two sealers. The cell viability at 24 hrs was 84%, at 72 hrs was 94.9% and on 7th day interval was 97.7%. Group 2 GuttaFlow bioseal sealer when compared to Group 1 AH Plus sealer showed significantly less cytotoxicity at all three intervals of time. Group 2 GuttaFlow bioseal sealer also showed less cytotoxicity than Group 3 Bio-C sealer however the difference in the cytotoxicity was not statistically significant at all three intervals of time. The cytotoxicity of the sealer showed significant reduction from slight to no toxicity (cell viability increased from 84% to 94.9%) from 24 hrs to 72 hrs and from 24 hrs to 7 days (cell viability increased from 84% to 97.7%). However, the reduction in cytotoxicity was not significant from 72 hrs to day 7 (cell viability increased from 94.9% to 97.7%).

The results of cytotoxicity of Group 2 GuttaFlow bioseal sealer are in accordance to previous cytotoxic analysis of the sealer.[1,3,22,23,24,26]

This higher biocompatibility property of Group 2 GuttaFlow bioseal sealer can be attributed to the presence of bioactive contents such as calcium silicate and also may be due to lack of resin content in its composition. [1,3,22,23,24,26] According to the animal study by Santos et al and Delfino et al on subcutaneous tissue of rat, there was an alkalinizing effect leading to accelerated tissue repair and mineralization due to release of bioactive ions (Ca^{+2} and OH^{-2}).[23,26] According to Rodríguez-Lozano et al,

the Group 2 GuttaFlow bioseal sealer has shown to promote cementoblastic differentiation of mesenchymal stem cells.[24]

The Group 3 Bioceramic sealer, Bio-C sealer showed significantly lower cytotoxicity than Group 1 AH Plus sealer at all time intervals, but showed higher cytotoxicity than GuttaFlow bioseal sealer, though the results were statistically insignificant. The cell viability at 24 hrs was 69.5%, at 72 hrs was 79.4% and on 7th day interval was 88.4 %. Though slight to moderate cytotoxic response was seen with this sealer, it is considered biocompatible due to its properties of having osteogenic potential, promotion of osteoblastic cell differentiation, release of bioactive ion and enhanced mineralization potential.[15,16,17,18,19]

The cytotoxicity decreased significantly from 24 hrs to 72 hrs (cell viability increased from 69.5% to 79.4%), from 72 hrs to 7th day (cell viability increased from 79.4% to 88.4%) from 24 hrs to 7th day (cell viability increased from 69.5% to 88.4%), the initial cytotoxicity may be attributed to the high alkalinity during setting reaction of the material.[15] These results are in accordance with the animal study conducted by Silva et al, where in initial inflammatory response was seen in rat subcutaneous tissue.[15] Thus, the Group 3 Bio-C sealer though slightly cytotoxic, has been said to be biocompatible.

Both Group 2 silicone based (GuttaFlow bioseal) sealer and Group 3 Bioceramic (Bio-C) sealer are considered to be biocompatible due to their superior physical and chemical properties, though they showed mild cytotoxicity at some interval of time. Thus, while selection of root canal sealer not only its biological behaviour but also various other parameters such as antimicrobial efficacy,

regenerative and healing properties and other physical and chemical properties should be considered.

Considering the limitations of this study, use of extended time interval of cytotoxicity, assessment of genotoxicity of the sealers and utilization of different assays for cytotoxicity evaluation should be carried out.

CONCLUSION

Considering the parameters of the study, the following conclusions were made: -

- Group 1 the Epoxy resin based, AH Plus sealer was proven to be the most cytotoxic among the three sealers at all three intervals of time i.e., at 24 hrs, 72 hrs and 7 days. Moderate cytotoxicity was reported at all intervals of time.
- Group 2 Silicone based, GuttaFlow bioseal was proven to be the least cytotoxic among the three sealers at all three intervals of time i.e., at 24 hrs, 72 hrs and 7 days. It showed slight to no cytotoxicity from 24 hrs to day 7.
- Group 3 Bioceramic sealer, Bio-C sealer was more cytotoxic than Group 2 GuttaFlow bioseal but was less cytotoxic than Group 1 AH Plus sealer. It showed slight cytotoxicity at all intervals of time.
- Both Group 2 Silicone based GuttaFlow bioseal and Group 3 Bio-C sealers are considered biocompatible though they showed mild cytotoxicity at some interval of time.

SUMMARY

The in-vitro study was conducted in the Department of Conservative Dentistry and Endodontics, KLE VK Institute of Dental Sciences, KLE Academy of Higher Education & Research (KLE University), Belagavi (Karnataka). Aim of this study was to evaluate and compare the cytotoxic effect of the Epoxy resin based, Silicone based and Bioceramic based endodontic sealers on human periodontal ligament fibroblasts by MTT assay.

The laboratory procedures required for cytotoxicity analysis using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay was carried out in KLE's Dr Prabhakar Kore Basic Sciences Research Centre, KLE University, Belagavi.

Excised PDL tissues from the extracted human maxillary and mandibular premolar teeth indicated for orthodontic extraction were placed in tissue culture dishes. Establishment of explant cultures was done. Subsequently sub-cultures were made and cell seedings were placed in respective wells in 96 well plates. Eighteen discs were fabricated from all the test groups which were Group1 - AH Plus sealer; Group2 – GuttaFlow bioseal sealer; Group 3 – Bio-C sealer. The cytotoxicity of the test materials was evaluated using MTT assay at 24 hrs, 72 hrs and 7 days interval. Viable cells in each well were calculated relative to control cells set to 100% and the absorbance was checked and recorded with 570nm using an ELISA microplate reader.

The percentage of cell viability was calculated using the formula[8]

$$\% \text{ of cell viability} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Cytotoxicity was rated based on cell viability relative to control as given by[8, 31:]

- Non cytotoxic (> 90% cell viability)
- Slightly cytotoxic (60 – 90% cell viability)
- Moderately cytotoxic (30 – 59% cell viability)
- Strongly cytotoxic (< 30% cell viability)

Statistical analysis was done using by one way ANOVA and Tukeys multiple posthoc procedures. The results showed that, Group 1 AH Plus sealer has proved to be the most cytotoxic at all three time intervals of 24 hrs, 72 hrs and 7 days among the three sealers, which reduced from 24 hrs to day 7. The Group 2 Silicone-based GuttaFlow bioseal sealer showed slight to no cytotoxicity over the three time intervals and showed the least cytotoxicity as compared to the other two sealers. Group 2 GuttaFlow bioseal sealer showed less cytotoxicity than Group 3 Bio-C sealer however the difference in the cytotoxicity was not statistically significant at all three intervals of time.

The study concluded that, both Group 2 silicone based (GuttaFlow bioseal) sealer and Group 3 bioceramic (Bio-C) sealer are considered to be biocompatible due to their superior physical and chemical properties, though they showed mild cytotoxicity at some interval of time.

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ANNEXURE I

ETHICAL CLEARANCE CERTIFICATE

Research and Ethics Committee
KLE V K INSTITUTE OF DENTAL SCIENCES
KLE University
Accredited 'A' Grade by NAAC Placed in Category 'A' by MHRD (GoI)
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Sl. No. : 1474

CERTIFICATE

This is to Certify that the synopsis titled

Comparative Evaluation of Cytotoxic Effect of Epoxy Resin-based, Silicone based & bio-ceramic based Endodontic sealers on human Periodontal ligament fibroblast by mtt assay - an in vitro study.

Submitted by Dr. _____ *P. G. Student / Staff, Guided by* _____ *from Department of* Department of Conservative dentistry & Endodontics *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 5/5/21

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

ANNEXURE II

BIOSTATISTICS CLEARANCE CERTIFICATE

KLE V.K. Institute of Dental Sciences
(A Constituent unit of KLE Academy of Higher Education & Research
Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi-590 010 INDIA
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Biostatistics Clearance Certificate

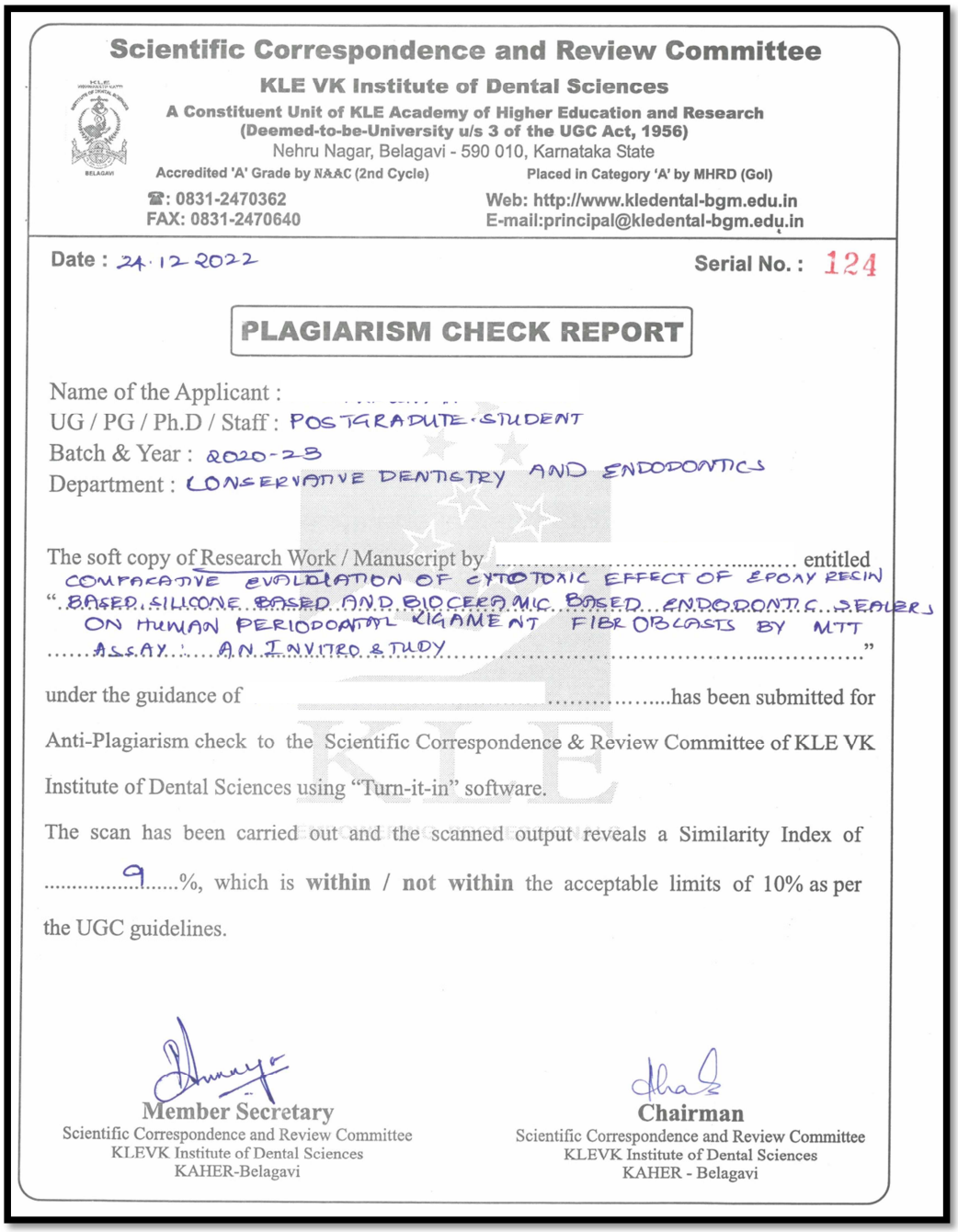
This is to certify that the Biostatistics aspect of the Dissertation / Research work **Post Graduate Student**, under the guidance of **Reader, Department of Conservative Dentistry and Endodontics** entitled "COMPARATIVE EVALUATION OF CYTOTOXIC EFFECT OF EPOXY RESIN-BASED, SILICONE BASED AND BIO-CERAMIC BASED ENDODONTIC SEALERS ON HUMAN PERIODONTAL LIGAMENT FIBROBLASTS BY MTT ASSAY: - AN IN-VITRO STUDY " has been done under my guidance and considered satisfactory.

Place: Belagavi
Date: 07/10/2022

Name & Signature of Biostatistician
(Dr. S. B. Javali)
USM KLE IMP
Belagavi. -10

ANNEXURE III

PLAGIARISM CHECK CERTIFICATE



Scientific Correspondence and Review Committee

KLE VK Institute of Dental Sciences

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PLAGIARISM CHECK REPORT

Name of the Applicant :
UG / PG / Ph.D / Staff : POSTGRADUTE STUDENT
Batch & Year : 2020-23
Department : CONSERVATIVE DENTISTRY AND ENDODONTICS

The soft copy of Research Work / Manuscript by ..... entitled
"COMPARATIVE EVALUATION OF CYTOTOXIC EFFECT OF EPOXY RESIN BASED, SILICONE BASED AND BIOCERAMIC BASED ENDODONTIC SEALERS ON HUMAN PERIODONTAL LIGAMENT FIBROBLASTS BY MTT ASSAY : AN INVITRO STUDY"

under the guidance of .....has been submitted for Anti-Plagiarism check to the Scientific Correspondence & Review Committee of KLE VK Institute of Dental Sciences using "Turn-it-in" software.

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Member Secretary
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KLEVK Institute of Dental Sciences
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Chairman
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