International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Biocompatibility of Silicate Based Root Canal Sealers on Human Fibroblast Cells-*In Vitro* Study based on Cell Viability Assay

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Abstract: ***Biocompatibility of any root canal sealer plays an important role in determiningthe outcome of the treatment. The recently launched silicate-based root canal sealers claim to have better biocompatibility compared to the regularly used sealers. In vitro study was done to compare the recently launched silicate-based root canal sealers (BioRoot RCS and Totalfill BC) with AH+ the "Gold standard" sealers used in clinical practice using cell viability assays and immunocytochemistry. Discs prepared from the respective sealers were placed with MM1 cell line (human gingival fibroblast) and WPS9 cell line (human periradicular fibroblast) for seven days to check the biocompatibility of these materials. The remaining live cells were either fixed with cold methanol for immunocytochemistry evaluation or lysed for proyein analysis. Cell viability was evaluated by 3-(4, 5-dimethylthiazole-2-yl)-2, 5-dipheyl tetrazolium bromide (MTT) assay using the material extracts collected at 24, 72 and 168 hours respectively. MTT assay demonstrated AH+ to be the most toxic followed by BioRot RCS and Totalfill BC.***

Keywords: Biocompatibility, Tricalcium silicate sealers, fibroblasts, AH+, MTT assay

1. Introduction

Portland cement happens to be the predecessor of these recently launched silicate-based sealers since most of their properties have been derived from Portland cement.

Historically it was Dr. Witte who was the first person to use Portland cement as a root canal filling [1]. Calcium silicate-based cement first came into the market in 1990 when MTA was introduced.

MTA is a tricalcium silicate based hydraulic powder which has been commercially used as a perforation repair and a root end filing material [2].

Various studies have been done to test the biocompatibility of calcium silicate-based materials which have yielded positive results [3]. This led to the idea of manufacturing a sealer which imbibed most of the properties of the calcium silicates and showed similar bioactivity when placed inside the root canal system.

When using any sealer there is a possibility of it being extruded into the peri radicular tissue can cause bio toxic reactions, but with the introduction of bioactive materials we believe that healing in the periapical area will occur more readily.

There appears to be a lack of number of peer reviewed papers establishing the effects of calcium silicates powders in root canal sealers in human clinical studies. All the studies that have been carried out are based on *in vitro* testing and *in vivo* animal models.

This study investigates the cytotoxic and bioactive effects of two calcium silicate-based bio ceramic sealers to gain a comprehensive understanding of the biological changes caused by these materials on human fibroblast cells.

2. Literature Review

An ideal root canal filling is the filling in which there is a "hermetic seal" which can be achieved by using both core material and a sealer in conjunction with one another.

Regardless of the material used, a sealer is the most essential component required to achieve a "fluid tight" seal [4].

The sealer is used as a lubricant during the obturation process, and it also fills in the voids and irregularities not only in the root canal but also in the lateral and accessory canals [4].

"Biocompatibility describes the ability of a material or a substance to perform with an appropriate host response when applied as intended."

"Cytotoxicity is defined as the capacity of the material to impact cellular viability. "

Before any material is launched into the market it must undergo several biocompatibility and cytotoxicity tests to determine the effects of these sealers. Almost all the sealers which are currently in use show a variable number of cytotoxic effects to the periapical tissues. The freshly mixed sealers happen to show the highest range of cytotoxic effects and it decreases with time [5] [6].

Calcium silicate-based materials were first introduced in 1993. Several studies have been conducted to test the stability, dye penetration, antibacterial effects, physiochemical properties and cytotoxicity of these materials since the first time they were introduced [7] [8] [9] [10] [11].

The handling qualities of these products were improvised, and the shortcomings were rectified based on several

experiments and this led to the development of improvised calcium silicate materials.

Clinical success can be assessed by the absence of symptoms, swellings or other signs of infections, evidence of radiographic healing and normal functioning of the tooth [12].

A study tested the biocompatibility of four different endodontic sealers with different bases on L929 mouse skin fibroblast cell line using multiscan Ex spectrophotometer. This study concluded that the methacrylate-based sealer was considered more cytotoxic than the other groups viz., silicone, epoxy resin, and calcium hydroxide-based sealers [13].

The cytotoxicity of AH+ and AH26 when AH+ was tested when they were launched. Cytotoxicity was assessed on a culture of human gingival fibroblast with the sealer extracts collected at different intervals. Findings concluded that AH+ were more biocompatible to the tissues than AH26 [14].

Contemporary root canal sealers like Roekoseal showed no cytotoxic effect when freshly mixed and when tested on 3T3 fibroblast after 3 days and 7 days. Cell viability was reduced when the same test conditions were applied for MTA Fillapex [15].

One Study evaluated the cytotoxicity of the root canal sealers Roekoseal and AH+ on human cervical carcinoma (HeLa) cells and mouse skin fibroblast (L929). This study showed that AH+ was slightly more toxic on both the cell lines while Roekoseal did not cause any significant changes in the cell line [16].

An experiment tested the cytotoxicity and osteogenic potential of AH+, Pulp canal sealer (ZOE based) and an experimental calcium silicate sealer on a MC3T3 osteogenic cell line at 6 weeks intervals. They found that all the sealers showed severe cytotoxicity after 24 hours and gradually reduced except the Pulp canal sealer. The experimental calcium silicate sealer was the least tissue irritating and never interfered with the bone regeneration [17].

Not many studies have been conducted on the newly launched BioRoot RCS and Totalfill BC sealers.

A study by evaluated the interaction of BioRoot RCS with human periodontal ligament cells. Comparison was made between BioRoot RCS and Pulp canal sealer were BioRootRcs was found to be less cytotoxic and induced higher secretion of angiogenic and osteogenic growth factor [18].

Cytotoxicity of Totalfill BC, MTA Fillapex and AH+ were compared by the assessment of their biological response on human periodontal ligament cells. Results showed that Totalfill BC was more biocompatible compared to the other two [19].

Since these sealers are very new in the market many studies are yet to be done on their biocompatibility. One cannot reach a conclusion based on in-vitro studies alone. By performing animal implantation experiment one can grasp the concept of cytotoxicity of calcium silicate-based sealers better.

Aims and Objectives

The main objective of this study was to compare the biocompatibility and changes in the cell morphology of the recently launched calcium silicate-based root canal sealers (BioRoot RCS abdTotalfill BC) with AH+ using direct cell viability assay and immunocytochemistry.

3. Materials and Methods

The root canal sealers that were used in the experiment are:

- **AH Plus-**This is the root canal sealers which is commonly used in Clinical practice. It is a resin-based sealer-Dentsply DeTrey, Konstanz, Germany.
- **Bioroot RCS –** Mineral based root canal sealer which is made from calcium silicate – Septodont, France.
- **Totalfill BC –** Mineral based root canal sealong material which has high alkaline property and exhibits zero shrinkage – Scottlander, Swizterland.

Cell Lines Used:

- **MM1 normal human oral mucosa fibroblast cells (donated by Dr. MichaelnaMacluskey, University of Dundee) –** harvested from the operculum of an impacted wisdom tooth of a 24-year-old healthy male donor during a surgical procedure. General ethical approval already exists for this cell line.
- **WPS9periradicular human fibroblast cells (donated by Professor Willam P Saunders, University Of Dundee) –** harvested from the bony crypt of a periradicular lesion of endodontic origin. The donor was a healthy 57-year-old non-smoker. All tissue within the bony crypt was excised as part of necessary treatment of the periradicular lesion and the patient was not subjected to additional procedures. As before, consent was gained from the patient and ethical approval was not required.

Cell Culture Reagents

- **Dimethyl sulphoxide (DMSO)** (SIGMA # D-5859, Sigma-Aldrich Company Ltd. Dorset, United Kingdom).
- **Ethanol** 70% in distilled water.
- **Foetal Calf Serum (FCS)** (SIGMA # D-5859, Sigma-Aldrich Company Ltd. Dorset, United Kingdom).
- **Hanks balanced salt solution (HBSS)** (SIGMA # H-4641, Sigma-Aldrich Company Ltd. Dorset, United Kingdom).
- **L-Glutamine** (SIGMA # G-7513, Sigma-Aldrich Company Ltd. Dorset, United Kingdom).
- **Methanol**-(SIGMA # 32213, Sigma-Aldrich Company Ltd. Dorset, United Kingdom).
- **Minimum Essential Medium Eagle (MEM)** (SIGMA # M-0275, Sigma-Aldrich Company Ltd. Dorset, United Kingdom).
- **Penicillin-Streptomycin stabilised (**SIGMA # P-4333, Sigma-Aldrich Company Ltd. Dorset, United Kingdom).
- **Phosphate buffered saline (PBS)** (SIGMA # P-4417, Sigma-Aldrich Company Ltd. Dorset, United Kingdom).

- **Pierce BCA protein assay kit** (THERMO-SCIENTIFIC # 23225, 23227, Lifetechnologies Ltd. Paisley, United Kingdom)
- **Tris (Tris (hydroxymethyl) aminomethane**) (SIGMA # 77-86-1, Sigma-Aldrich, Company Ltd. Dorset, United Kingdom)
- **Triton X-100 (4-(1, 1, 3, 3-Tetramethylbutyl) phenyl-polyethylene glycol)** (SIGMA # 9002-93-1, Sigma-Aldrich Company Ltd. Dorset, United Kingdom).
- **Tween 20 (Polysorbate 20)** (SIGMA # P9416, Sigma-Aldrich Company Ltd. Dorset, United Kingdom).
- **Trypsin** (SIGMA # T-4549, Sigma-Aldrich Company Ltd. Dorset, England).
- **3-(4, Dimethylthiazzol-2yl)-2, 5-diphenyltetrazolium bromide (MTT)** (SIGMA # M-2128, Sigma-Aldrich Company Ltd. Dorset, United Kingdom).

Determination of effects of material leachable products on cell viability

To test the effect of leachable products of the mineral based root canal sealers, three pellets/ discs of the rootcanal sealers were placed in 25ml of universal dish filled with serum free MEM growth media. These serum free extracts were collected at an interval of 24 hours, 3 days and 7 days. This extract was used for MTT assay to determine cell viability.

MTT Assay

The MTT (3 (4, 5-dimethylthiazol-2-yl)-2, 5-dipenyltetrazolium bromide) colorimetric assay was originally described by Mosmann. This is used as the nonradioactive quantification of cellular proliferation, viability and cytotoxicity. It is based on the ability of living cells to reduce tetrazolium salts into coloured formazan compounds. This reduction takes place only when mitochondrial reductase enzymes are active and therefore considered to be a measurement of viable cells.

Plating of wells

Three 90mm dishes of MM1 and WPS9 each were taken in a class II biological cabinet and the confluence were checked. The growth media was ejected out and the cells were washed with 5ml of Hanks balanced solution twice. These dishes were trypsinised after adding 2 ml of trypsin. This was kept incubator for 5 minutes. After the cells are detached they are neutralised using 2ml of 10% FCS MEM growth media and collected in the universal jar. The cells were collected using a 10ul pipette and placed on a Flurostar Optima plate reader and are counted immediately using BioRADTC10 automated Cell Counter. Calculate the volume of cells required to plate the density, 500ul cell suspension per well. This plate was kept in the incubator overnight for attachment.

$$\textit{Volume of cell solution} = \frac{\textit{Optimised cell density x number of wells x volume per well}}{\textit{required for experiment cell density of single cell suspension}}$$

The required volume of single cell suspension was transferred to a centrifuge tube and centrifuged in a bench top centrifuge at 900 rpm for 5 minutes to produce a cell pellet. The supernatant was then aspirated off the cell pellet.

Then required amount of growth media (10% FCS) is added to the cell suspension and it is suspended thoroughly. After this 500µl of cell suspension is placed into each well of the 48 well plate leaving behind the last two rows which are blank.

The following table (1) shows the plan for the plating of cells for MTT assay before the cells are exposed to the test conditions.

	1	2	3	4	5	6	7	8
A	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS
B	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS
C	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS
D	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS	$2x10^4$cells in 10% FCS
E	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
F	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

The serum free extract which was extracted from the material is used to make test conditions viz., 5%, 2%, 1% which is plated added to the 48 well plate.

Exposure of fibroblast to material eluates.

Four 48 well plates were used per cell line (MM1 and WPS9) and per eluate sample collection period (1 day, day3 and day7).

This table (2) shows the number of 48 well plate required to carry out the MTT assay.

Material used	Baseline	Day 1	Day 3	Day 7
AH+	1	1	1	1
Bioroot RCS	1	1	1	1
Totalfill BC	1	1	1	1

One out of the four 48 well plate for each cell line (MM1 and WPS9) is taken and base line count is done. Test conditions made from the material eluate serum free (5%, 2%, 1%, SF) are added to the 48 well plate after checking the cell attachment to the wells. MTT assay is done with the remaining three 48 well plate on day1, day3 and day7.

The table (3) shows the plan which was devised to get results after the cells are exposed to material extracts.

	1 day extract	1 day extract	3 days extract	3 days extract	7 days extract	7 days extract	Control	Control
A	Cells+ 5% FCS made from SF extract	Cells+ 5% FCS made from SF extract	Cells+ 5% FCS made from SF extract	Cells+ 5% FCS made from SF extract	Cells+ 5% FCS made from SF extract	Cells+ 5% FCS made from SF extract	Cells+ 5% FCS	Cells+ 5% FCS
B	Cells+ 2% FCS made from SF extract	Cells+ 2% FCS made from SF extract	Cells+ 2% FCS made from SF extract	Cells+ 2% FCS made from SF extract	Cells+ 2% FCS made from SF extract	Cells+ 2% FCS made from SF extract	Cells+ 2% FCS	Cells+ 2% FCS
C	Cells+ 1% FCS made from SF extract	Cells+ 1% FCS made from SF extract	Cells+ 1% FCS made from SF extract	Cells+ 1% FCS made from SF extract	Cells+ 1% FCS made from SF extract	Cells+ 1% FCS made from SF extract	Cells+ 1% FCS	Cells+ 1% FCS
D	Cells+ SF extract	Cells+ SF extract	Cells+ SF extract	Cells+ SF extract	Cells+ SF extract	Cells+ SF extract	Cells+ SF	Cells+ SF
E	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
F	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

MTT Assay for 48 well plate

The MTT assay count is done for baseline, day1, day3 and day7.

All these procedures are carried out in the class II biological cabinet. The MTT solution should always be stored in the dark because of its photosensitive nature.

Cells attachment is checked in each well and then the media is tapped out gently onto paper towels. The wells are washed gently twice with serum free MEM, 500ul per well.

Add MTT solution to give a final concentration of 1mg/ml. place the plate in the incubator at 37^{o}C for 3 hours. During the incubation time the yellow MTT is reduced to purple formazan in the mitochondria of any living cells attached to the plate.

Remove MTT solution from wells by tapping the contents on to some paper tissues. Add 500ul of DMSO (Dimethyl sulfoxide) per well. Place the plate on the shaker for 20mins at room temperature. The DMSO will dissolve the formazan, creating a coloured solution.

The resulting-coloured solution was assessed by reading the plate spectrophotometrically on Flurostar Optima plate reader at 540nm. References filter 620nm.

Data conversion and normalisation process

Data from all days are compared and using Microsoft Excel 2013 to identify significant differences between treatment groups.

$$\text{Normalised data value} = \frac{\text{Absorbance value of treatments x 100}}{\text{Mean absorbance of control cells}}$$

4. Results

MM1 gingival fibroblast and WPS9periradicular fibroblast were used in eluates exposure experiments. Both MM1 gingival fibroblast and WPS9periradicular fibroblast were plated at a cell density of 2 x 10^4 cells ml^{-1}. All the eluate dilutions, negative controls and optical blanks and optical controls were supplemented with 10% FCS.

MM1 gingival fibroblasts exposed to varying degree of eluate concentration.

MM1 gingival fibroblasts were exposed to varying degree of eluate concentration (5% of FCS, 2% of FCS, 1% of FCS, Serum Free eluates) of AH Plus, Bioroot RCS, Totalfiil BC for over a period of 168 hours, with MTT assay being conducted at 24, 72 and 168 hours respectively to assess the cell viability. Serum free media without any material eluate was considered as the control for all groups.

Exposure of MM1 fibroblasts to eluates of AH plus

Eluates collected after 1 day:

There was an increase in the cell viability in all the eluate concentration. Almost two-fold increase in the cell viability was noted in the 5% FCS eluate concentration on the first day count, which remained constant on the third and the seventh day. When comparing the 2% FCS eluate concentration, the cell viability was almost two times compared to the control on the first day count. This reduced slightly on the third day count and increased more than threefold on the seventh day count.

Similarly for 1% of FCS eluate concentration the cell viability increased double that of the control on the first day count. But it decreased by 10% less than the control on the third day count followed by a marked threefold increase on the seventh day. In case of serum free eluate, the cell viability was similar to the control on the first day counting, then it saw a huge dip in the cells on the third day and a considerable increase on the 7th day.

Eluates collected after three days:

The cell viability of all eluate concentration (5%, 2%, 1%, Serum Free) was like the readings recorded for the eluate concentration collected after 24 hours on the first day count.

There was slight decrease in the cell viabily in all eluate concentrations on the third day count with the serum free showing the least cell viability which was almost half that of the control.

After this it was noted that there was a steady increase in the cell viability in all eluate concentration on the seventh day count.

Eluates collected after seven days

When the comparison of cell viability was done the 5% FCS eluate concentration showed a marked increase on the first day count. The growth was steady throughout when the third day and the seventh day counts were taken.

Similarly, for both 2% eluate concentration and 1% eluate concentration there was a steady growth of cells on day one, three and seven. In case of 1% eluate concentration there was slight deterioration of growth on the third day which was insignificant.

When serum free eluate concentration was considered, there was a steady decrease of the cell viability which became almost nil on the day three count after which there was a steady increase.

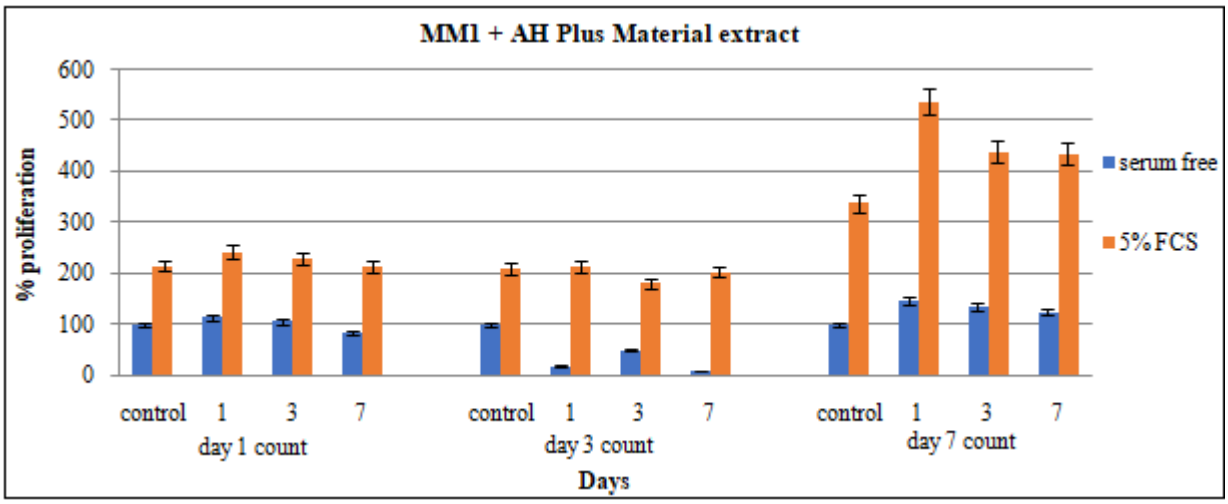

Figure 1: Effect of AH plus materials on the proliferation of MM1 cells. AH plus material extracts were collected at different time point and used them in the MTT assay to investigate the effect of material on cell's proliferation.1= material extracts collected after 1 day with serum free or 5% FCS media, 2= material extracts collected after 3 days with serum free or 5% FCS media and 3= material extracts collected after 7 days with serum free or 5% FCS media. Control (Ctrl) denotes to serum free and 5% FCS media only without extracts.

Exposure of MM1 fibroblasts to eluates of Bioroot RCS

Eluates collected after one day:

There was an increase in the cell viability in all the eluate concentration except the serum free eluate. In the 5% FCS eluate group there was not much increase in the cell viability in first day count but there was almost two fold increase in the cell viability noted on the third day count and the it was similar on the seventh day count as well. When comparing the 2% FCS eluate concentration, the cell viability was almost on par with the control group on the first day count. This increased slightly on the third day count and remained constant on the seventh day count.

Similarly for 1% of FCS eluate concentration the cell viability kept on fluctuation 5% to 10% above and below the control group on all three days. In case of serum free eluate, the cell viability was almost less than 20% in comparison to the control on the first day counting, then it saw a steady increase in the cells on the third and seventh day.

Eluate collected after three days.

In case of the 5% FCS eluate concentration, it was slightly higher than the control group on the first day count and the cell viability increased almost two times by the third day count and there was steady increase in the cell viability which was noted on the seventh day count.

When the 2% FCS eluate concentration was considered cell viability was on the higher side when compared to the control group on the first day count, then there was a twofold increase in the cells on the third day count followed by a small dip in the cell viability on the seventh day count.

Similarly for 1% FCS eluate concentration there was no marked difference in cell viability in all the three days.

Cell viability in case of serum free eluate was almost 60% less than the control group on the first day count which reduced further on the third day with no remarkable increase on the seventh day.

Eluate collected after seven days.

When the comparison of cell viability was done with the 5% FCS eluate concentration, it showed readings like that of the control group on the first day. The growth was almost twofold on the third and the seventh day.

Similarly for the 2% eluate concentration the cell viability was almost same as that of the control group on the first day with a steady increace on the third day and slight decrease on the seventh day. In case of 1% eluate concentration there was minimal deterioration of growth on first day followed by steady increase on the third and seventh days.

When serum free eluate concentration was considered, there was a marked decrease of the cell viability on the first day which keeps deteriorating on the third and the seventh days.

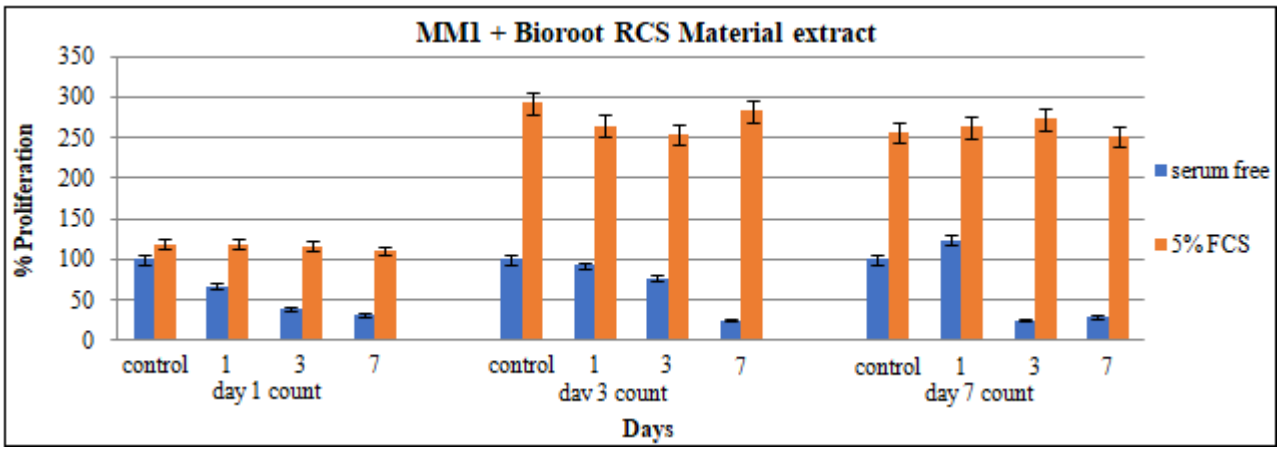

Figure 2: Effect of Bioroot RCS materials on the proliferation of MM1 cells. Bioroot RCS material extracts were collected at different time point and used them in the MTT assay to investigate the effect of material on cell's proliferation.1= material extracts collected after 1 day with serum free or 5% FCS media, 2= material extracts collected after 3 days with serum free or 5% FCS media and 3= material extracts collected after 7 days with serum free or 5% FCS media. Control (Ctrl) denotes to serum free and 5% FCS media only without extracts.

Exposure of MM1 fibroblasts to eluates of Totalfill BC

Eluates collected after one day:
There is a steady increase in the cell viability in case of off the all-eluate concentration (5% FCS, 2% FCS, 1% FCS and Serum free) on all the three days count. Serum free eluates concentration happened to have the closest value of cell viability to that of the control group.

Eluates collected after three days:
There is decrease in the cell viability count in case of 5% FCS eluate concentration on the first day followed by a gradual increase and a slight dip in the third and seventh days respectively.
When cell viability of 2% FCS eluate concentration is considered there is a gradual increase on the first and the third day with a minor decrease on the seventh day. In case of 1% FCS and serum free eluate concentration there is a gradual increase in the cell viability on all three days.

Eluates collected after seven days:
Cell viability count in 5% FCS eluate concentration showed twofold increase on the third day count compared to the first day count followed by a decrease in the cells on the seventh day.

There is a gradual increase in the cell viability in case of 2% FCS eluate concentration on the first and the third day count and a negligible decrease on the seventh day count.

Cell viability count in case of 1% FCS eluate concentration is similar to that of the control group on the first day after which it increases on the third day and decreases again on the seventh day.

There is a steady increase in the serum free eluate concentration when compared to the control group on all three days.

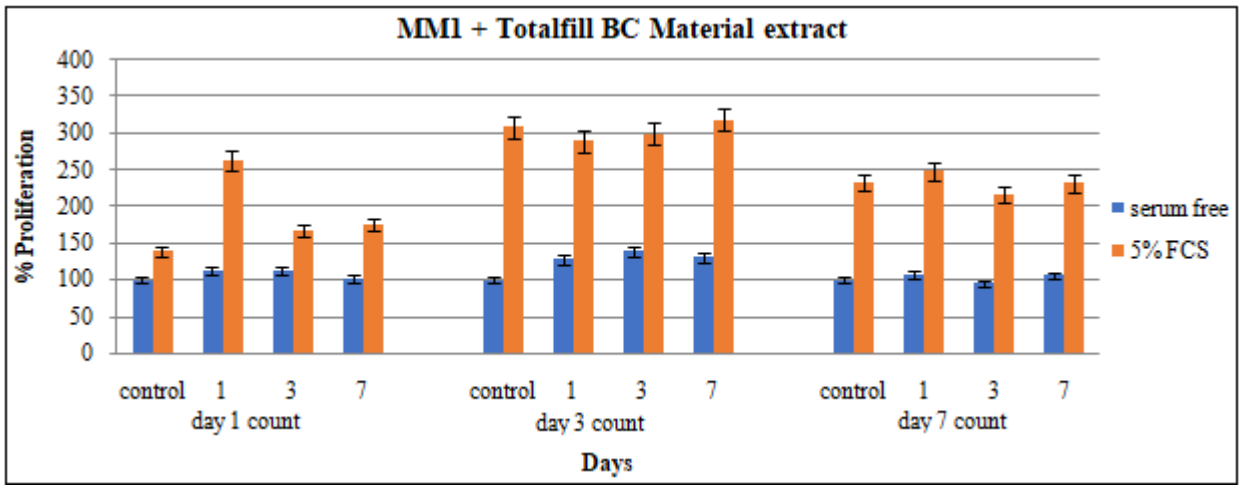

Figure 3: Effect of Totalfill BC materials on the proliferation of MM1 cells. Totalfill BC material extracts were collected at different time point and used them in the MTT assay to investigate the effect of material on cell's proliferation.1= material extracts collected after 1 day with serum free or 5% FCS media, 2= material extracts collected after 3 days with serum free or 5% FCS media and 3= material extracts collected after 7 days with serum free or 5% FCS media. Control (Ctrl) denotes to serum free and 5% FCS media only without extracts.

WPS9periradicular fibroblasts exposed to varying degree of eluate concentration.

WPS9periradicular fibroblasts were exposed to varying degree of eluate concentration (5% of FCS, 2% of FCS, 1% of FCS, Serum Free eluates) of AH Plus, Bioroot RCS, Total fiil BC for over a period of 168 hours, with MTT assay being conducted at 24, 72 and 168 hours respectively to assess the cell viability.

Serum free media without any material eluate was considered as the control for all groups.

Exposure of WPS9 fibroblasts to eluates of AH Plus

Eluates collected after one day:

Taking into consideration about the cell viability there is a marked increase in the cell viability on the first and the third day followed by a sudden fivefold decrease on the seventh day in case of all the eluate concentrations (5% FCS, 2%FCS, 1% FCS and Serum free).

Eluates collected after three days:

Similar trend is followed in all the eluate concentrations. There is a slight decrease followed by stupendous increase on the first and the third day respectively. Then there is again a huge dip in the cell viability on the seventh day.

Eluates collected after seven days:

Again, the cell viability count follows a similar curve in respect to all the concentrations. Increase in cell viability is seen on the fist and the third day and sudden decrease in the cell viability is noted on the seventh day.

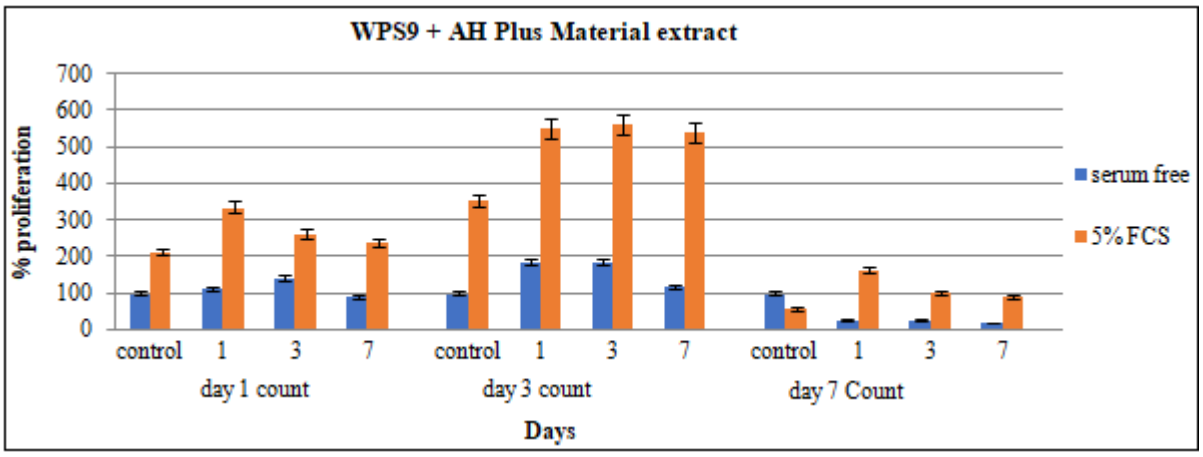

Figure 4: Effect of AH+ materials on the proliferation of WPS9 cells. AH plus material extracts were collected at different time point and used them in the MTT assay to investigate the effect of material on cell's proliferation.1= material extracts collected after 1 day with serum free or 5% FCS media, 2= material extracts collected after 3 days with serum free or 5% FCS media and 3= material extracts collected after 7 days with serum free or 5% FCS media. Control (Ctrl) denotes to serum free and 5% FCS media only without extracts.

Exposure of WPS9 fibroblasts to eluates of Bioroot RCS

Eluates collected after one day:

The cell viability shows increases on the first and becomes three times more on the third day followed by a slight decrease on the seventh day in case of all the eluate concentrations (5% FCS, 2%FCS, 1% FCS and Serum free).

Eluates collected after three days:

A uniform shift is seen in all the eluate concentrations. There is a slight increase compared to the control group followed by twofold increase on the first and the third day respectively. Then there is again a drastic dip in the cell viability on the seventh day.

Eluate collected after seven days:

Cell viability happens to be constantly increasing respect to 5% FCS and 2% FCS eluate concentration on the first and the third days.

In respect to 1% FCS and serum free eluate concentration there is gradual increase of cell viability on the first day followed by minimal decrease on the third day and a gentle increase on the seventh day.

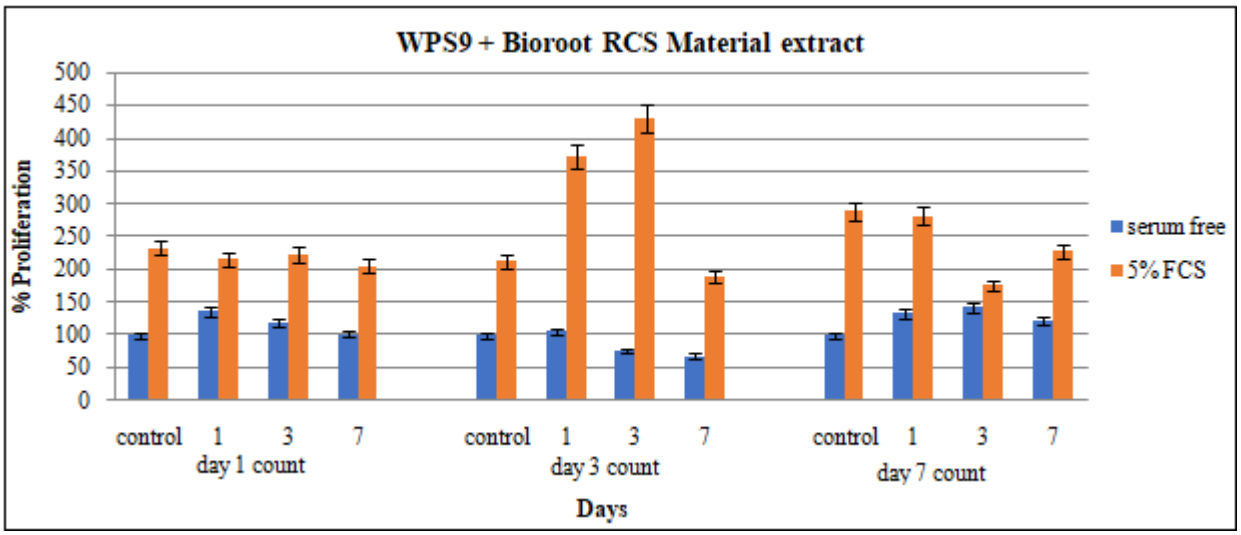

Figure 5: Effect of Bioroot RCS materials on the proliferation of WPS9 cells. Bioroot RCS material extracts were collected at different time point and used them in the MTT assay to investigate the effect of material on cell's proliferation.1= material extracts collected after 1 day with serum free or 5% FCS media, 2= material extracts collected after 3 days with serum free or 5% FCS media and 3= material extracts collected after 7 days with serum free or 5% FCS media. Control (Ctrl) denotes to serum free and 5% FCS media only without extracts.

Exposure of MM1 fibroblasts to eluates of Totalfill BC

Eluates collected after one day:
There is a steady increase in the cell viability in the 5% FCS eluate concentration on all three days. Constant decrease in the cell viability is noted in case of all the other eluate concentration (2% FCS, 1% FCS, Serum free) on all three days.

Eluates collected after three days:
A consistent increase in the cell viability is shown in the 5% FCS and serum free eluate concentration on all three days. The cell viability appears to be minimal in respect to 2% FCS eluate concentration on the first and the third day and no changes are noted on the seventh day. Uniform decrease in the cell viability is noted in relation to the 1% FCS eluate concentration.

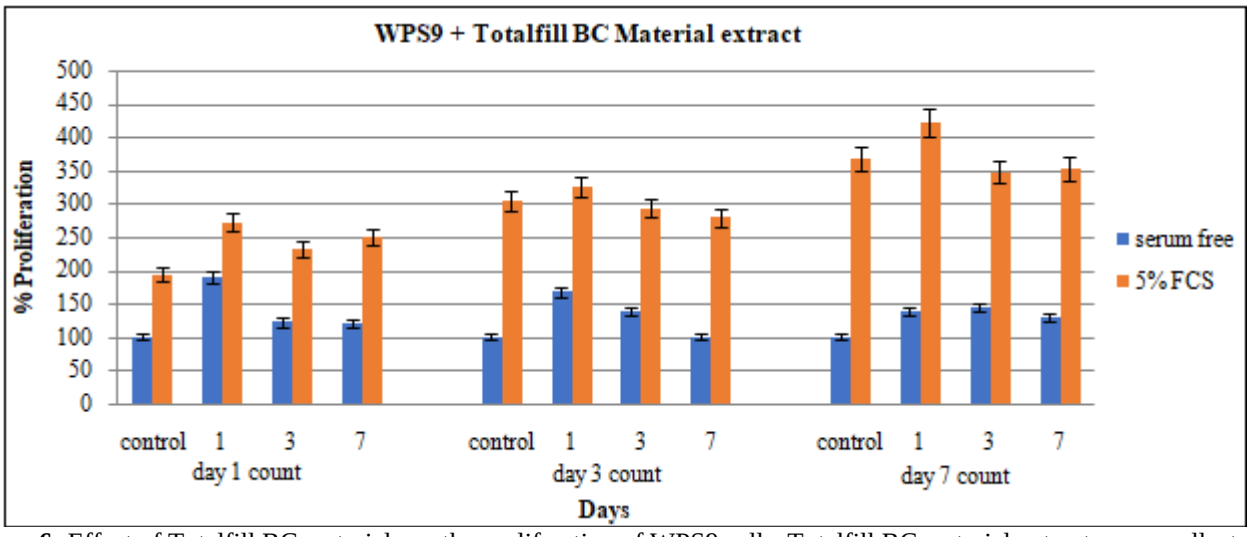

Figure 6: Effect of Totalfill BC materials on the proliferation of WPS9 cells. Totalfill BC material extracts were collected at different time point and used them in the MTT assay to investigate the effect of material on cell's proliferation.1= material extracts collected after 1 day with serum free or 5% FCS media, 2= material extracts collected after 3 days with serum free or 5% FCS media and 3= material extracts collected after 7 days with serum free or 5% FCS media. Control (Ctrl) denotes to serum free and 5% FCS media only without extracts.

5. Discussion

Method of eluate collections have varied between each researcher especially in terms of eluate extraction time and extraction ratios [17] [20].

The most used method for testing the cytotoxicity of a material in vitro is by using the MTT (3 (4, 5-dimethylthiazol-2-yl)-2, 5-dipenyltetrazolium bromide) colorimetric assay because it is relatively in expensive and easy to perform [21]

Earliest known experiment to determine the cytotoxic effect of AH Plus was done by compared AH Plus with its predecessor AH 26 using DNA-intercalating Fluorescent dye on primary human periodontal fibroblast and permanent 3T3

monolayers. As mentioned previously AH Plus was found to be superior to AH 26 which maybe because of reduced formaldehyde release [22].

A neutral red cytotoxicity assay compared AH Plus, AH 26 and Zinc oxide eugenol sealers on human gingival fibroblast. AH Plus was found to be the least cytotoxic. This result does not correspond with the result of this study because we found AH Plus to be most toxic of all the other sealers tested. The results of the earlier experiments cannot be emphasised on because AH Plus was compared to potentially more cytotoxic sealers in those experiments and the usage of tri calcium silicate cements was not widespread [14].

AH Plus was reported to have the highest toxicity in the first 24 hours and then the toxicity decreases gradually over time and almost becomes non-existent after 6 weeks [17]. This was not noted in this project because there was exponential decrease in the cell viability in presence of AH Plus on the seventh day, this can be due to the toxicity derived from the epoxy part of AH Plus which is releases after setting [22].

AH Plus was also compared with Gutta Flow (silicone based sealer), Apexit (calcium hydroxide based sealer), Endorez (methacrylate based sealer) on L929 mouse permanent fibroblast where it was found that AH Plus was biocompatible compared to the methacrylate-based sealer [13].

This finding can be correlated with the fact that mouse fibroblast was used instead of human fibroblast in this study. There is a high chance for the sealers to show different expressions when in contact with human fibroblasts.

Another study compared the cytotoxicity of RC Sealer, Epiphany, EndoREZ, GuttaFlow, Acroseal, AH Plus, RoekoSeal and Apexit on human gingival fibroblasts and mouse fibroblast cell line L929. It was found that AH Plus did not exert any cytotoxic effect to the human gingival fibroblasts and induced bell proliferation in L929 mouse fibroblast cell line. This is not in accordance with this study and using different test conditions could have been possible cause [24].

A study evaluated the cell viability AH Plus, Endomethasone N, Pulp Canal Sealer EWT or Sealapex using tryphan blue assay on human fibroblasts and it correlates with the results of our study where it was found that AH Plus is the comparatively more toxic than the other sealers used. The only difference in the tryphan blue assay is that freshly mixed materials are plated on to culture plates to check the viability of the cells rather than using the eluates [25].

As suggested before, it should be noted that different researchers prefer to use different methods to check for cytotoxicity.

When comparing the cytotoxic effect of AH Plus with Endorez BC a bioceramic sealer, it was found that the cytotoxicity and the genotoxiciy of AH plus was more when compared to Endosequence BC [17]. This can be correlated with the results obtained in this project as the test materials used were also bioceramic based sealers.

This project states that the biocompatibility of the Bioroot RCS material is not the best because it caused morphological changes in the cells and cell viability also reduced after 7 days. Bioroot RCS when compared with Pulp canal sealer (Zinc oxide eugenol based sealer) showed better bioactivity with regard to human periodontal ligament cells [18]. This result maybe because of different material used for comparison.

Comparison of the biocompatibility of three calcium silicate based sealers (Bioroot BC, Endoseal MTA and Nano ceramic sealer) on human periodontal ligament stem cells. Out of the three Bioroot BC and Nano ceramic sealer showed better biocompatibility. This again corresponds with the result of our project partially [27].

Study compared the cytotoxic effect of 8 root canal sealers on human gingival fibroblasts. AH Plus showed no cytotoxic effect in the first 24 hours, moderate effect after 48 hours and severe cytotoxicity after 72 hours. In case of Bioroot RCS and Totalfill BC it has been noted that the cytotoxic effect is mild to moderate throughout the 72 hours. This can be well correlated with the results obtained in our experiment [19].

Comparison of biocompatibility of Totalfill BC with AH Plus and MTA fillapex on human periodontal ligament cells showed that Totalfill BC has the best biocompatibility which is very similar to the results obtained in this project [28].

Despite various studies presenting the side effects of AH Plus it still happen to be the most used endodontic sealer worldwide. It has to be noted that in order to get stable results it is necessary to mimic the oral environment. This is relatively hard in case of in vitro studies. It should always be considered that the material which is cytotoxic in vitro need not always show the same result when placed clinically [16].

When oral environment is considered, we should always bear in mind that their presence of inflammatory cells and macrophages which can handle the changes caused by the sealers. But the extent of damage that these sealers produce is what that is to be examined.
Further studies regardingTotalfill BC and Bioroot RCS should be done to know further about these materials.

6. Conclusion

Principle findings of this study include:
The epoxy resin sealer AH+ was partially toxic to both gingival and periradicular fibroblasts.

7. Limitation of this study

Many studies have suggested that the toxicity of various root canal sealers is the maximum when freshly mixed and decreases over six weeks. It will be worth to study the materials in a larger scale with a bigger time frame to see if the results coincide with the other studies.

References

[1] Camilleri, J., 2017. Will Bioceramics be the Future Root Canal Filling Materials?. Current Oral Health Reports, 4 (3), Pp.228-238.

[2] Torabinejad, M. and Parirokh, M., 2010. Mineral trioxide aggregate: a comprehensive literature review—part II: leakage and biocompatibility investigations. *Journal of endodontics*, *36* (2), pp.190-202.

[3] Gandolfi, M. G., Taddei, P., Tinti, A. and Prati, C., 2010. Apatite-forming ability (bioactivity) of ProRoot MTA. *International Endodontic Journal*, *43* (10), pp.917-929.

[4] *Hargreaves, K. M. and Berman, L. H., 2015. Cohen's pathways of the pulp. Elsevier Health Sciences.* (no date).

[5] Shah, N. *et al.* (2015) 'Biotoxicity of commonly used root canal sealers: A meta-analysis', *Journal of Conservative Dentistry*, 18 (2), p.83. doi: 10.4103/0972-0707.153054.

[6] Javidi, M. *et al.* (2017) 'In vitro cytotoxicity of a new nano root canal sealer on human gingival fibroblasts', *Iranian Endodontic Journal*, 12 (2), pp.220–225. doi: 10.7508/iej.2017.02.018.

[7] Torabinejad, M., Higa, R. K., McKendry, D. J. and Ford, T. R. P., 1994. Dye leakage of four root end filling materials: effects of blood contamination. *Journal of endodontics*, *20* (4), pp.159-163.

[8] Torabinejad, M., Hong, C. U., Ford, T. P. and Kettering, J. D., 1995. Cytotoxicity of four root end filling materials. *Journal of endodontics*, *21* (10), pp.489-492.

[9] Torabinejad, M., Rastegar, A. F., Kettering, J. D. and Ford, T. R. P., 1995. Bacterial leakage of mineral trioxide aggregate as a root-end filling material. *Journal of endodontics*, *21* (3), pp.109-112.

[10] Koh, E. T., Torabinejad, M., Pitt Ford, T. R., Brady, K. and McDonald, F., 1997. Mineral trioxide aggregate stimulates a biological response in human osteoblasts. *Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials and The Japanese Society for Biomaterials*, *37* (3), pp.432-439.

[11] Torabinejad, M., Ford, T. R. P., Abedi, H. R., Kariyawasam, S. P. and Tang, H. M., 1998. Tissue reaction to implanted root-end filling materials in the tibia and mandible of guinea pigs. *Journal of Endodontics*, *24* (7), pp.468-471.

[12] Johnson, B. R., 1999. Considerations in the selection of a root-end filling material. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology*, *87* (4), pp.398-404.

[13] Konjhodzic-Prcic, A., Jakupovic, S., Hasic-Brankovic, L. and Vukovic, A., 2015. Evaluation of biocompatibility of root canal sealers on L929 fibroblasts with multiscan EX spectrophotometer. *Acta Informatica Medica*, *23* (3), p.135.

[14] Azar, N. G., Heidari, M., Bahrami, Z. S. and Shokri, F., 2000. In vitro cytotoxicity of a new epoxy resin root canal sealer. *Journal of endodontics*, *26* (8), pp.462-465.

[15] Silva, E. J. N. L. D., Santos, C. C. and Zaia, A. A., 2013. Long-term cytotoxic effects of contemporary root canal sealers. *Journal of Applied Oral Science*, *21*, pp.43-47.

[16] Miletić, I., Devčić, N., Anić, I., Borčić, J., Karlović, Z. and Osmak, M., 2005. The cytotoxicity of RoekoSeal and AH plus compared during different setting periods. *Journal of Endodontics*, *31* (4), pp.307-309.

[17] Bryan, T. E., Khechen, K., Brackett, M. G., Messer, R. L., El-Awady, A., Primus, C. M., Gutmann, J. L. and Tay, F. R., 2010. In vitro osteogenic potential of an experimental calcium silicate–based root canal sealer. *Journal of endodontics*, *36* (7), pp.1163-1169.

[18] Camps, J., Jeanneau, C., El Ayachi, I., Laurent, P. and About, I., 2015. Bioactivity of a calcium silicate–based endodontic cement (BioRoot RCS): interactions with human periodontal ligament cells in vitro. *Journal of endodontics*, *41* (9), pp.1469-1473.

[19] Poggio, C., Riva, P., Chiesa, M., Colombo, M. and Pietrocola, G., 2017. Comparative cytotoxicity evaluation of eight root canal sealers. *Journal of Clinical and Experimental Dentistry*, *9* (4), p. e574.

[20] Zhao, X., He, W., Song, Z., Tong, Z., Li, S. and Ni, L., 2012. Mineral trioxide aggregate promotes odontoblastic differentiation via mitogen-activated protein kinase pathway in human dental pulp stem cells. *Molecular Biology Reports*, *39*, pp.215-220.

[21] Shen, Y. A., Peng, B., Yang, Y., Ma, J. and Haapasalo, M., 2015. What do different tests tell about the mechanical and biological properties of bioceramicmaterials? *Endodontic Topics*, *32* (1), pp.47-85.

[22] Genotoxicity and cytotoxicity of the epoxy resin-based root canal sealer AH plus.

[23] Huang, F. M., Lee, S. S., Yang, S. F. and Chang, Y. C., 2009. Up-regulation of Receptor Activator Nuclear Factor–Kappa B Ligand Expression by Root Canal Sealers in Human Osteoblastic Cells. *Journal of Endodontics*, *35* (3), pp.363-366.

[24] Eldeniz, A. U., Mustafa, K., Ørstavik, D. and Dahl, J. E., 2007. Cytotoxicity of new resin-, calcium hydroxide-and silicone-based root canal sealers on fibroblasts derived from human gingiva and L929 cell lines. *International endodontic journal*, *40* (5), pp.329-337.

[25] Silva, E. J., Neves, A. A., De-Deus, G., Accorsi-Mendonça, T., Moraes, A. P., Valentim, R. M. and Moreira, E. J., 2015. Cytotoxicity and gelatinolytic activity of a new silicon-based endodontic sealer. *Journal of applied biomaterials & functional materials*, *13* (4), pp.376-380.

[26] Candeiro, G. D. M., Moura-Netto, C. D., D'Almeida-Couto, R. S., Azambuja-Júnior, N., Marques, M. M., Cai, S. and Gavini, G., 2016. Cytotoxicity, genotoxicity and antibacterial effectiveness of a bioceramic endodontic sealer. *International endodontic journal*, *49* (9), pp.858-864.

[27] Collado-González, M., García-Bernal, D., Oñate-Sánchez, R. E., Ortolani-Seltenerich, P. S., Lozano, A., Forner, L., Llena, C. and Rodríguez-Lozano, F. J., 2017. Biocompatibility of three new calcium silicate-based endodontic sealers

on human periodontal ligament stem cells. *International endodontic journal*, *50* (9), pp.875-884.

[28] Rodríguez-Lozano, F. J., García-Bernal, D., Oñate-Sánchez, R. E., Ortolani-Seltenerich, P. S., Forner, L. and Moraleda, J. M., 2017. Evaluation of cytocompatibility of calcium silicate-based endodontic sealers and their effects on the biological responses of mesenchymal dental stem cells. *International endodontic journal*, *50* (1), pp.67-76.