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Biocompatibility of Silicate Based Root Canal Sealers on Human Fibroblast Cells - *In Vitro* Study Based on Direct Visual Observation

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Abstract: ***Tricalcium silicate sealers which have been launched on the market claim to have better biocompatibility compared to other sealers which are regularly used. In vitro study was conducted to compare the biocompatibility and cytotoxicity of the recently launched calcium silicate-based root canal sealers (BioRoot RCS and Totalfill BC) with AH+ which is commonly used in the clinical practice and is the "gold standard" using direct visual observations. 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. Visual observations shows that AH+ sealer is the most toxic as there is an increased amount of cell death with respect to both the cell lines and the tricalcium sealers caused more morphological changes in MM1 when compared to WPS9.***

Keywords: Biocompatibility, Tricalcium silicate sealers, fibroblasts, AH+, visual observation.

1. Introduction

Bio ceramic sealers are the most recently launched sealers in the market. They are calcium silicate based and 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 looks into 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 amount 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].

AH+ is an epoxy resin-based sealer which has been used in our clinical setting for a a considerable amount of time given its usage in three dimensional (3D) gutta percha compaction techniques. This sealer has been developed after modifying certain qualities of its predecessor AH26, which was successfully been in use for the past 50 years.

AH26 was known to discolour tooth structure and release formaldehyde. AH+ eliminated these problems. AH+ is said to have good radio opacity which is one of the essential criteria to be fulfilled by a good endodontic sealer. Good

dimensional stability along with tissue compatibility certifies its prolonged use in the clinical practice[7] [8].

Added advantage of AH+ was its easy removal from the rot canal system in case of re root canal treatment [9].

BioRoot RCS (Septodont, Saint MAur-des Fosses, France) is a new water-based sealer composed of tricalcium silicate, zirconium oxide and povidone that is available in the market since 2015 [11] [12]. It is shown to form a calcium phosphate phase when in contact with a physiological solution [12]. Studies claimed that this cement facilitated formation of hydroxyapatite at the tooth sealer interface which in turn caused the mineralization of the dentinal structures [13].

Totalfill BC (Schottlander, Switzerland) is another novel bioceramic sealer which requires presence of water (present in the dentin) to set and harden. Its composition is aluminium free, and it's said to have excellent physical properties with minimal dimensional shrinkage when placed in the root canal system.

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 visual observation.

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.

Determination of effects of material on cell morphology

To test the viability of the cells, the cells were cultured in 35mm sterilised plastic petri dishes. They were allowed to reach a confluency of 40% - 50%. The materials were then placed in these dishes and the changes were noted using light microscopy. Changes were noted at an interval of 7 days.

Material sample preparation

Custom 8 x 1mm cylindrical moulds were made from 1mm thickness polytetrafluoroethylene (PTFE) sheet drilled to produce 8mm diameter holes.This was then supported by another two 1mm PTFE sheet on either side, and the whole assembly bolted to a 2 or 3mm stainless steel plate drilled and tapped to accept M4 stainless steel bolts on both sides.

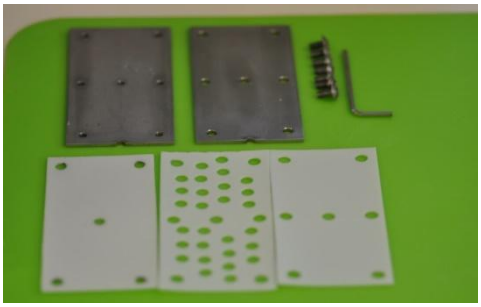


Figure 1: Construction of custom moulds for production of material disc

Materials were mixed in a class II biological cabinet as per manufacturer's instructions. The material was then placed on the 8 x 1 mm PTFE moulds and the assembly was screwed tightly as mentioned before. This was then kept inside the incubator at 37°C overnight for setting. This procedure was followed for the preparation of AH+ and BioRoot RCS samples.

As mentioned by the manufacturers the Totalfill BC required a wet field provide by the dentinal fluid for setting. For sample preparation, to provide a wet environment for the product, wet paper towels were places inside a 90mm petri dish and few deops of sealers were placed in the middle pf the dish. This petri dish was kept inside at 37^{o}C overnight for setting. This process was also followed inside the class II biological cabinet.

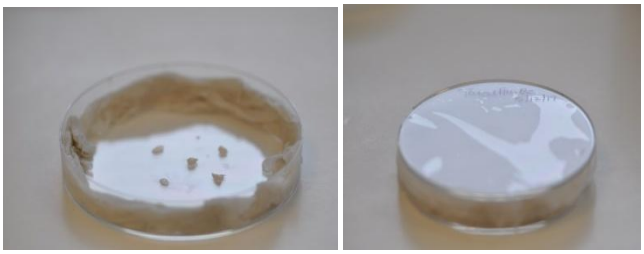


Figure 2,3: Totalfill BC sealer placed on a 90mm petri dish with wet paper towels.

After the materials are set the assembly is unscrewed in the class II biological cabinet and the materials were removed and placed in a sterilised 90mm petri dish. The materials were placed in the UV [14] cabinet using a sterilized metal forceps and sterilised for 2 minutes at 3200 Joules energy on either side. The materials were again placed in another sterile 90 mm petri dish and sterilised for 2 minutes. These materials were stored in a clean and dry place and used when needed.

Cell culture procedure

Oral fibroblasts were obtained from the cell bank of the Department of Cell and Molecular Biology, School of Dentistry, University of Dundee, and cultured on 90mm plastic petri dishes. Cells were incubated in a cell incubator at a constant 37°C, 95% humidity and 5% CO_2 in 7ml of normal growth medium (MEM supplemented with 10% heat inactivated foetal calf serum and 1% L-glutamine (20 mmol/l)). (Schor *et al.*, 2003). The growth media was changed every 24-48 hours until the cells were confluent. Cultures were then passaged using trypsin (tyrpsinisation).

The 90 mm petri dish was opened in class II biological cabinet and the media was aspirated. The petri dish was then washed with 5 ml of Hank's balanced solution twice. 2ml of trypsin/EGTA was then added and placed in the incubator for 5 minutes. The dish was taken out and checked if the cells are detached. Almost 90% of the cells were detached. 2ml of growth media (containing 10% FCS) was added to neutralise the trypsin. The cell suspension was collected in one universal container and centrifuged in a bench top centrifuge at 900 rpm for 5 minutes to produce a cell pellet. The supernatant media was aspirated, and 2 ml of fresh media was added. 5ml of media plus 2ml of cell suspension were added to a 90 mm petri dish and kept in the incubator at a constant 37°C, 95% humidity and 5% CO_2 in 7ml of normal growth media.

Growth medium was changed every 72-96 hours in a Class II biological cabinet, and then returned to the cell incubator.

Visual Observation

One 90mm petri dish was taken and farmed into three 60mm dishes as mentioned in 1.2.2. These 60mm dishes were kept inside the incubator for overnight for cells to attach. Three pellets of root canal sealer sample discs were taken and each of them was placed in the 60mm dishes. These three dishes were placed inside the incubator overnight to check the reaction of the cells to the material.

These dishes were observed under microscope for the time of 24 hours, 3 days, and 7 days. Changes were noted in the fibroblast and these changes were photographed using SM50 microscope camera.

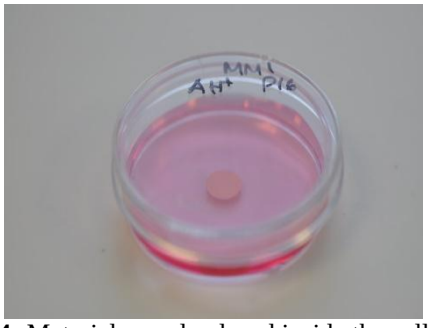


Figure 4: Material sample placed inside the cell culture plate

These dishes were observed under microscope for the time of 24 hours, 3 days, and 7 days. Changes were noted in the fibroblast and these changes were photographed using SM50 microscope camera.

4. Results

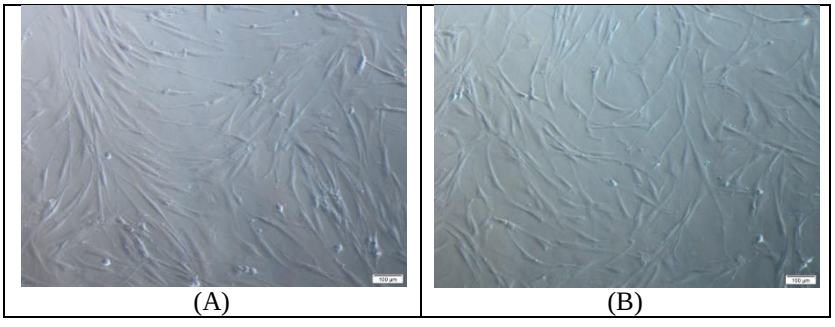
Culture of MM1 Fibroblast for 7 days in presence of AH+, Bioroot RCS and Totalfill BC discs

MM1 fibroblasts were cultured in 35 mm plastic petri dishes after which a disc of each material (AH+, Bioroot RCS, Totalfill BC) was placed in the dish. The cells were monitored for 7 days to check for any morphological changes caused due to the presence of the material disc. To aid in the observation photomicrographs were taken for each material and cell changes were recorded.

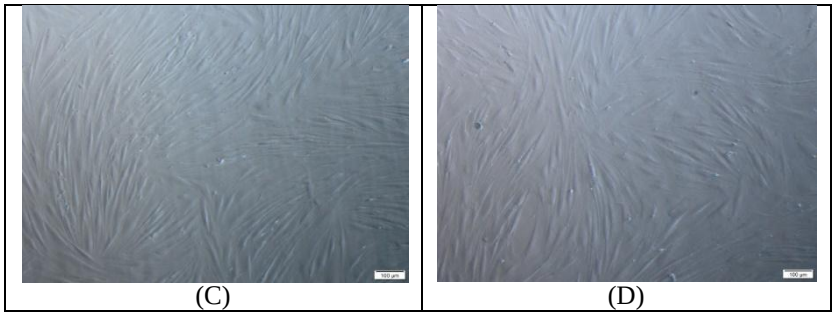
Photomicrographs were taken primarily in three different areas for assessment.One near the immediate vicinity of the material sample, one was taken slightly away from the sample and another one was taken remote to the sample, in addition to this if there were any other changes noticed, they were also recorded.Change of in cell morphology, reduction in cell density was considered.

MM1 gingival fibroblast control cell culture

On the first day there was medium density of the cells. The confluence of the dish was around 40% (complete confluence was not yet achieved) *(A).* There were some spaces which were clearly seen in between the cells. The cells were multipolar and had normal cytoplasmic processes. This was a marked increase in the cell density the following days and by the 7^{th} day the cells were completely confluent and were more aligned with a bipolar appearance*(D).*



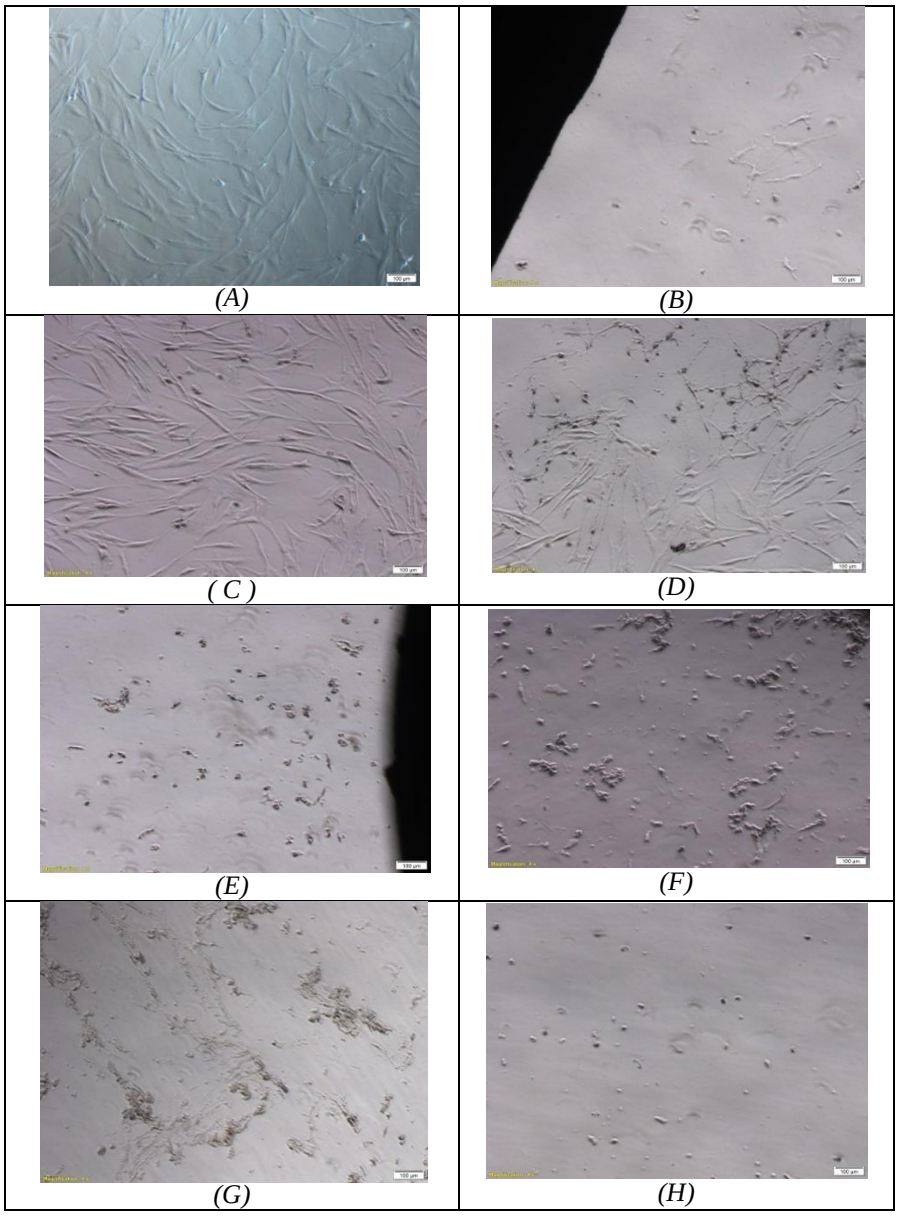
(A) (B)



(C) (D)

Figure 5: (A) (B) shows 30% - 40% confluence with gaps in between the cells on day 1 and day 3. Complete confluence on day 6 and day 7 is shown in (C) and (D).

MM1 gingival fibroblasts cultured with AH Plus sample. Changes were evident when AH Plus sample was placed in the cell culture dish which was compared to the control dish without sample *(A)*.On the first day almost one third cells present in two dishes showed cell death. This was mostly concentrated on the area in the close vicinity of the material sample*(B).* There were some live cells present away from the material sample (*C).* There was no notable difference between the cells on the second day *(D)*. On the third day we could see considerable changes in the cells in all three dishes. Almost 95% of the cells were dead and the remaining few cells were extremely stressed and showed signs of dying *(F)*. On the fourth and the fifth day almost all the cells were dead in all the three dishes *(G,H).*



(A) *(B)*

(C) *(D)*

(E) *(F)*

(G) *(H)*

Figure 6: MM1 cells response to AH+ is shown to be drastic with the dead cells near the material on the 1st day (B) and cells changing shape throughout till 4th day (D, E, F). Complete cell death is seen on the 5th day (H).

MM1 gingival fibroblast cultured with Bioroot RCS samples

MM1 fibroblast appeared to be normal on the first day, in both the areas close and remote to the material sample. It was noted that there was leaching of some material from the sample which formed a precipitate around the sample *(A)*. It was noted that there was leaching of some material from the sample which formed a precipitate around the sample *(B)*. On the second and the third day the cells close to the material showed slight changes in shape and the cells away from the material appeared to be stressed *(D, E)*. Considerable changes in the shape and density of the cells were noted in the sixth day. There was marked increase in the leaching of material and there was a zone of dead cells present near the material. The cells appeared to have changed shape throughout; they appeared to be more rounded *(H)*. The same was observed in the seventh day as well, except that there was more reduction in the cell density *(I, J)*.

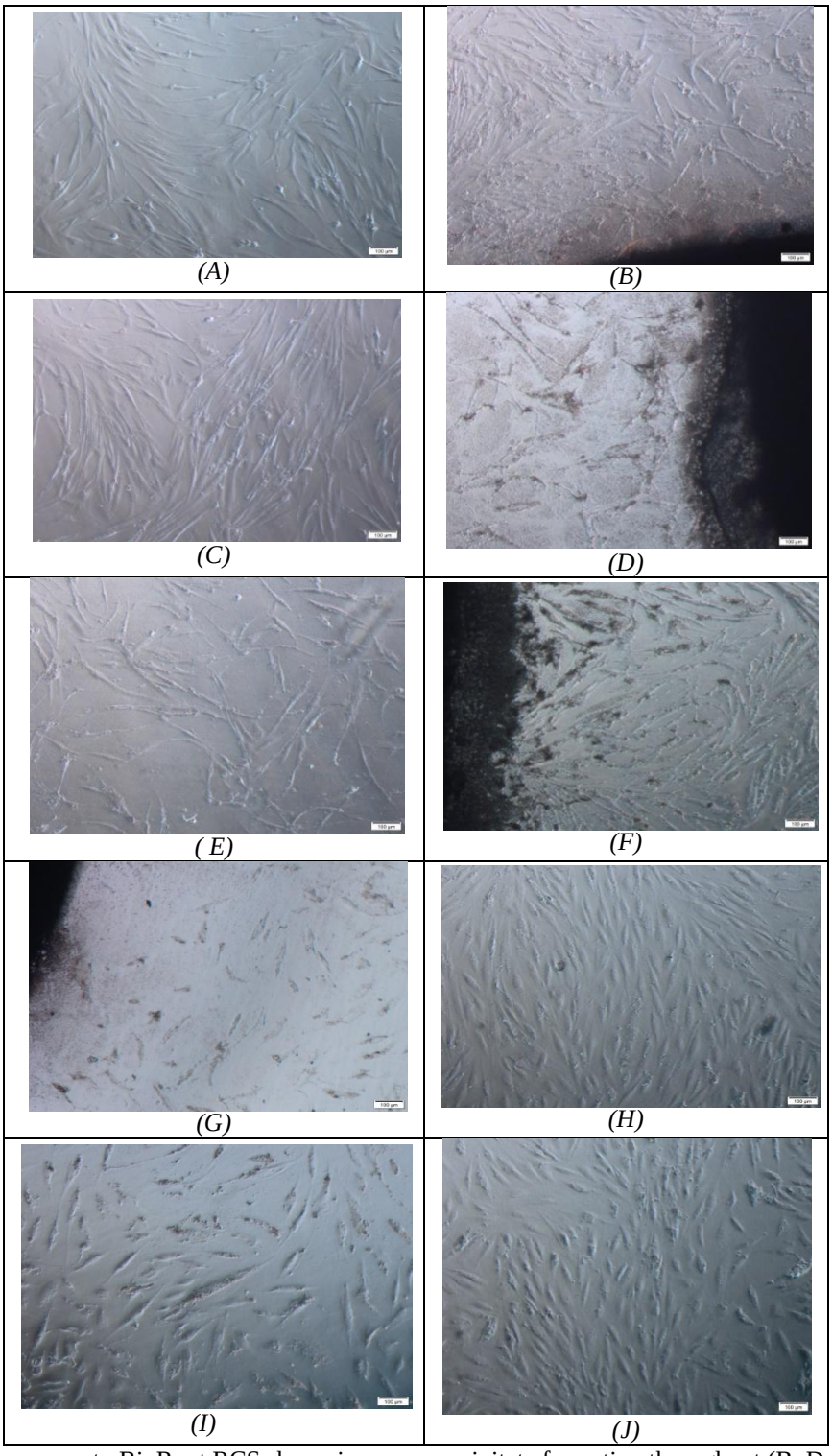
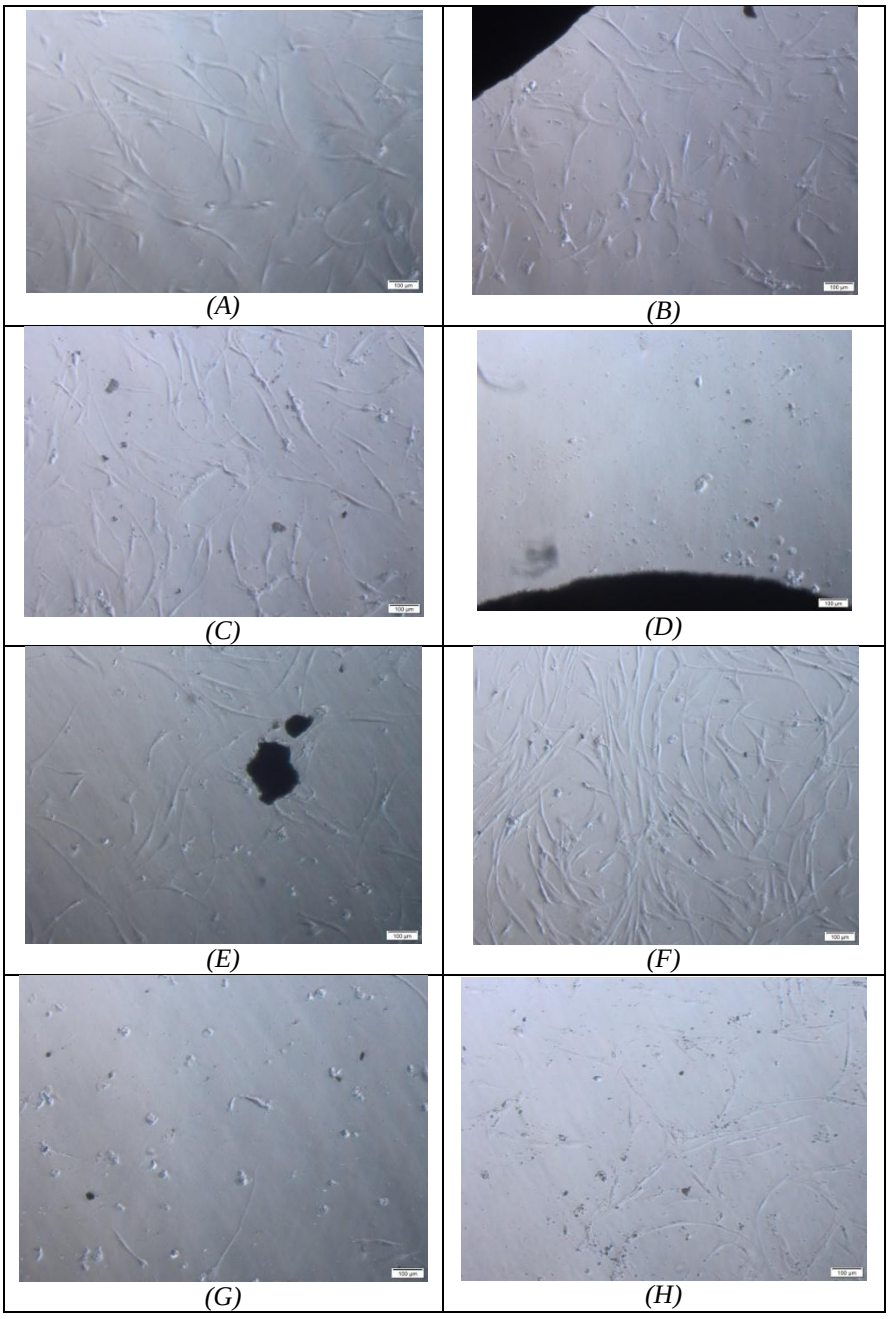


Figure 7: MM1 cells response to BioRoot RCS shows increase precipitate formation throughout (B, D, F). There is reduction in cell density from the 6th day (G). Morphological changes in the cells can be noted (H, I, J)

MM1 gingival fibroblasts cultured with Totalfill BC samples

Changes to the MM1 gingival fibroblast were not very evident on the first day after placement of Totalfill BC samples when compared to the control dish without material sample (A). No clear changes were observed in the cells close to the material sample (*B)* and the cells remote to the sample appeared to be healthy *(C).* On the second day the material appeared to have changed its position and there were few fragments of material left behind where the material was first placed, there was a zone of dead cells near these fragments (*E)*

Cells near the material appear to be slightly stressed (*H)* while the cells away from the material were healthy *(F)*. No marked changes were noted on the third day. On the sixth day cells near the material appeared to be slightly stressed and there was an area of dead cells near the material *(K)*. Morphological changes to the cells near the material fragments are also noted *(I)*. Cells away from the material appear to be healthy. There no remarkable difference seen on the seventh day. There is slight leaching of material and there is a zone of dead cells near the leached material. Cells away from the material show some morphological changes, but it is not uniform *(L, M, N).*



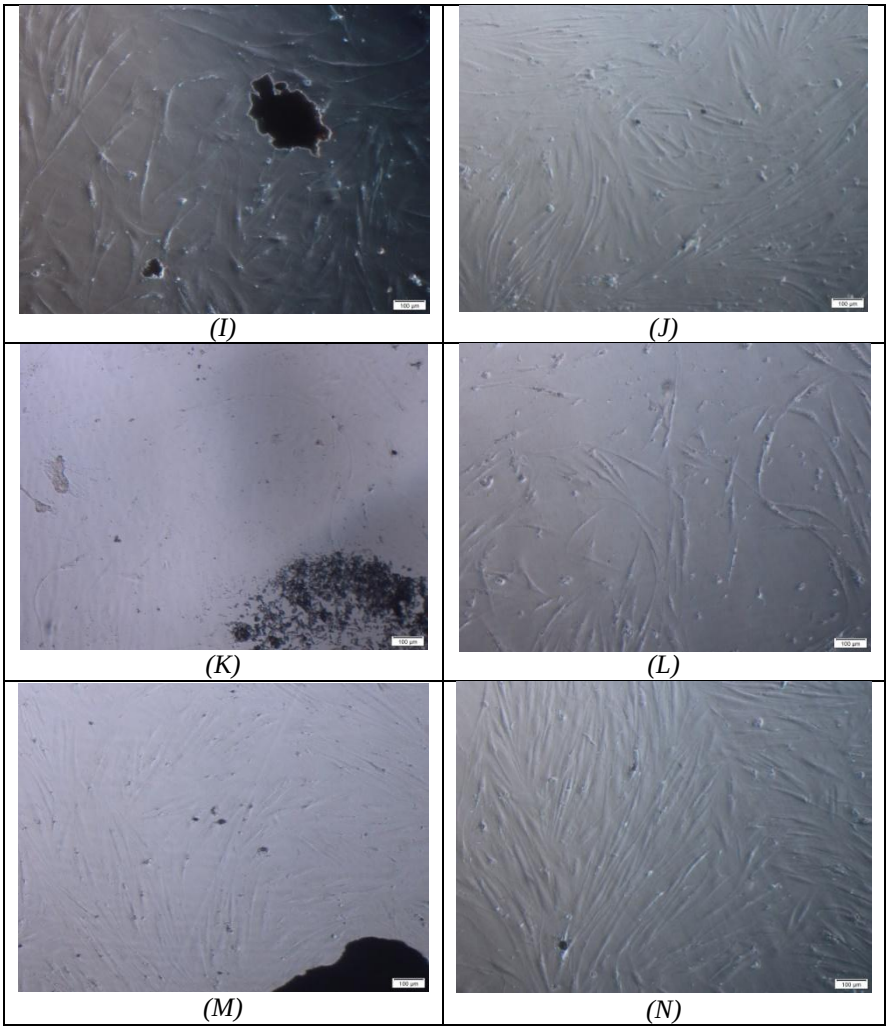


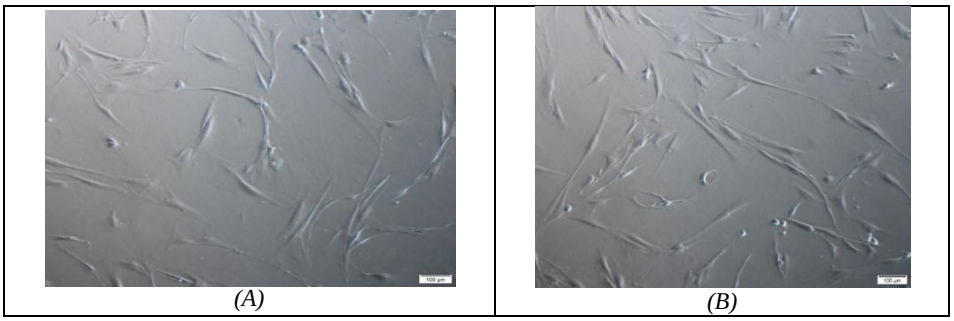
Figure 8: MM1 cells response to Totalfill BC is less drastic with cell death seen only in few areas (D, E, G, H, K). Cells are healthy away from the material till the third day (C, F) and are stressed on the 7th day (L, N).

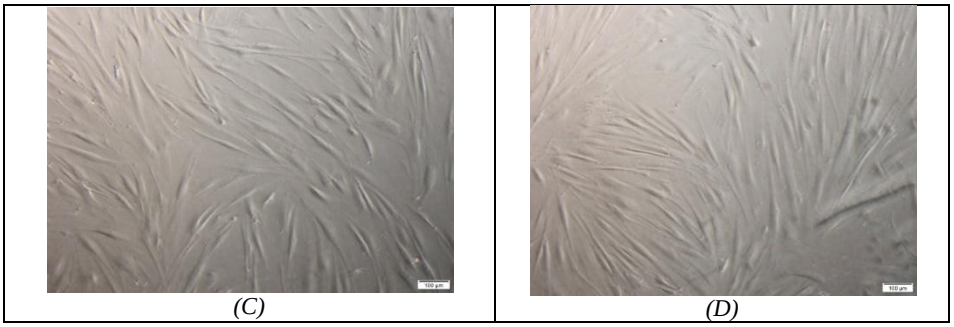
Culture of WPS9Periradicular Fibroblast for 7 days in presence of AH+, Bioroot RCS and Totalfill BC discs.

The WPS9periradicular fibroblasts were cultured in the presence of the materials (AH+, Bioroot RCS, Totalfill BC). This was done similar to the MM1 gingival fibroblast cells as mentioned above. Photomicroraphs of the cells were taken and any noticeable changes recorded.

WPS9Periradicular Fibroblast control cell culture.

WPS9 cell culture has sparse cell growth as compared to the MM1 cell culture. The density of the cells varied in the control dishes. The cells were 40% confluent by the fourth day and had multipolar appearance. By the 7th day complete confluence was achieved, with the cell having more tiled and bipolar appearance.



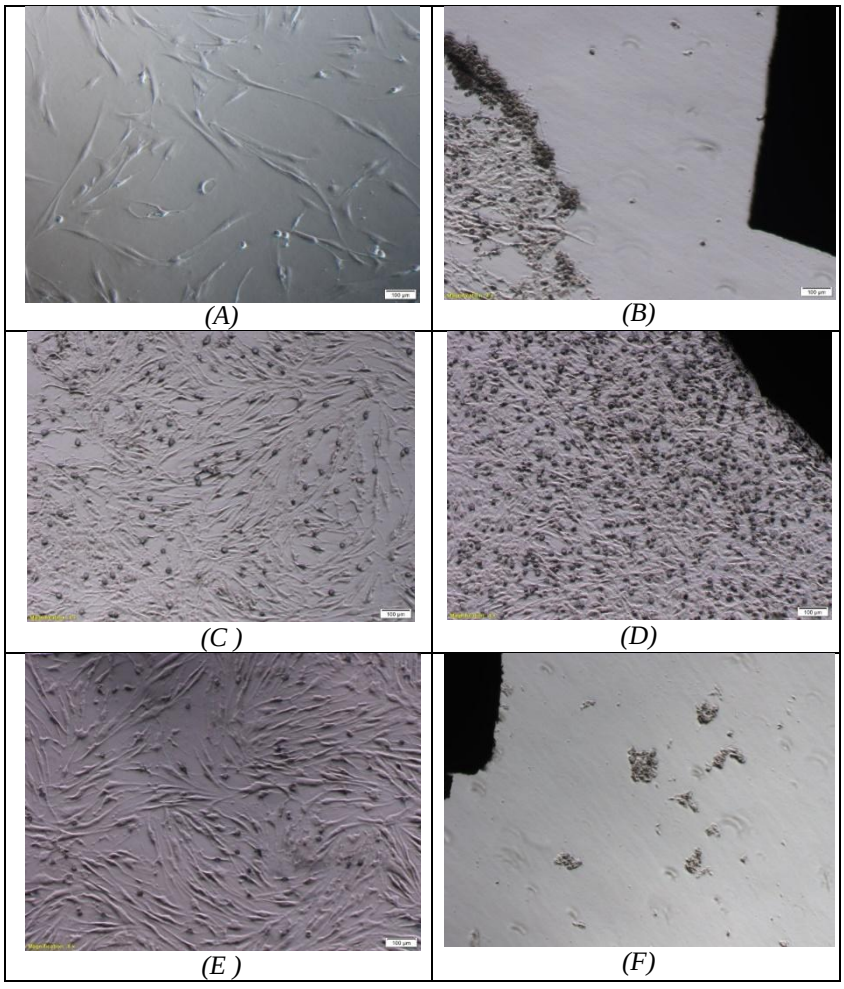


(C) *(D)*

Figure 9: (A) and (B) show 30% - 40% confluence with gaps in between the cells on day 1 and day 3. Complete confluence on day 6 and day 7 is shown in (C) and (D)

WPS9periradicular fibroblasts cultured with AH Plus samples.

Evident changes were seen after AH Plus sample was placed in the cell culture dish. This was compared to the control dish without samples *(A).* On the first day almost one third cells present in two dishes showed cell death. Area of dead cells was mostly concentrated on the area in the close vicinity of the material sample (*B).* There were some live cells present away from the material sample (*C).* There was difference in the cell density in the dishes, one of the dishes had over confluent cells. Even though there was cell death present near the material it in this dish, it was camouflaged by the over confluent cells (*D).*There was no notable difference between the cells on the second day. On the third day almost 95% of the cells were dead and the remaining few cells were extremely stressed and showed signs of dying *(F).* On the fourth and the fifth day almost all the cells were dead in all the three dishes *(G,H).*



(A) *(B)*

(C) *(D)*

(E) *(F)*

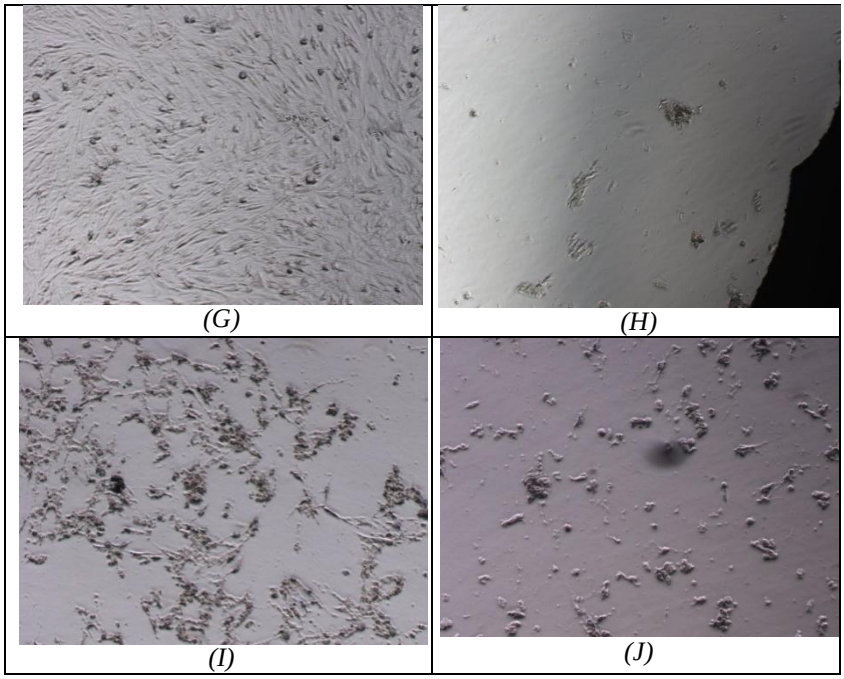
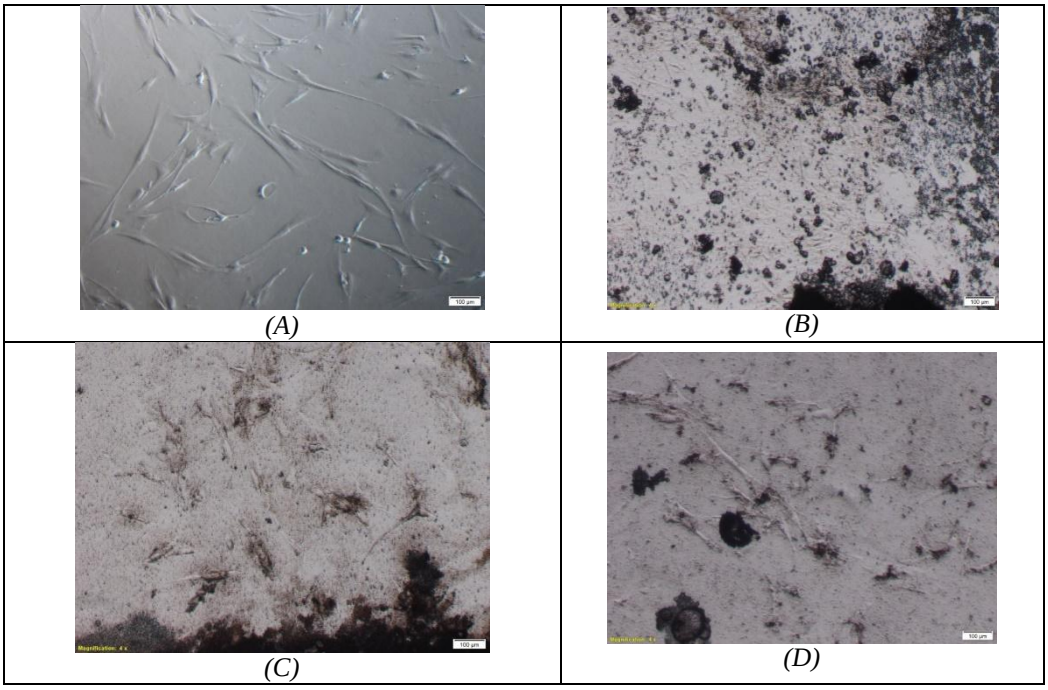


Figure 10: WPS9 cells response to AH+ is drastic as there is 30% - 40% cell death on the 1st day (B, C, D) which increases to 50% - 60% on the 3rd and 4th day (F, G, H. I) complete cell death is seen on the 5th day (J).

WPS9periradicular fibroblasts cultured with Bioroot RCS samples

A dish containing only cells in 10% FCS with no material sample was taken as a control (*A*).WPS9 fibroblasts tolerated BioRoot RCS well for the first two days There were no evident changes in the cell morphology even though there was marked release of precipitate from the material sample except for a few areas where the cells appear to be stressed *(B, C, D)*. Changes in the cell density was noted in the fifth day where the cells were multipolar, since the precipitate from the sample was relatively more it was difficult to appreciate the changes in the cells (*E, F).* On the seventh day it was noted that the cells were stressed near the material (*G)* and slight changes in the morphology of the cells were seen in the cells away from the materials *(H).*



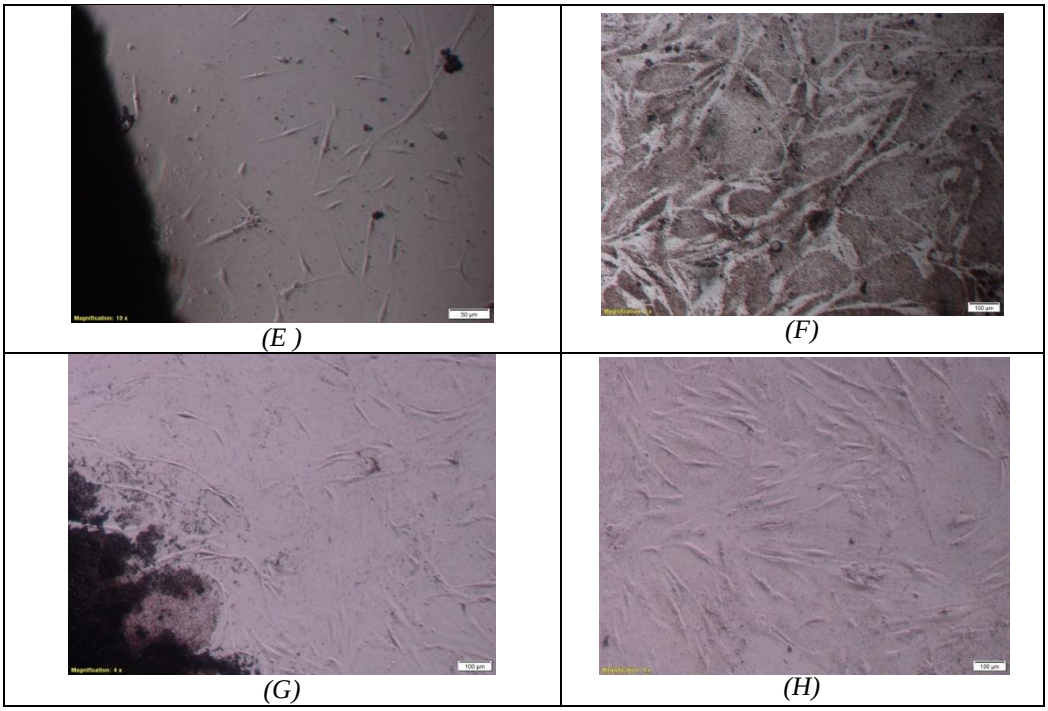
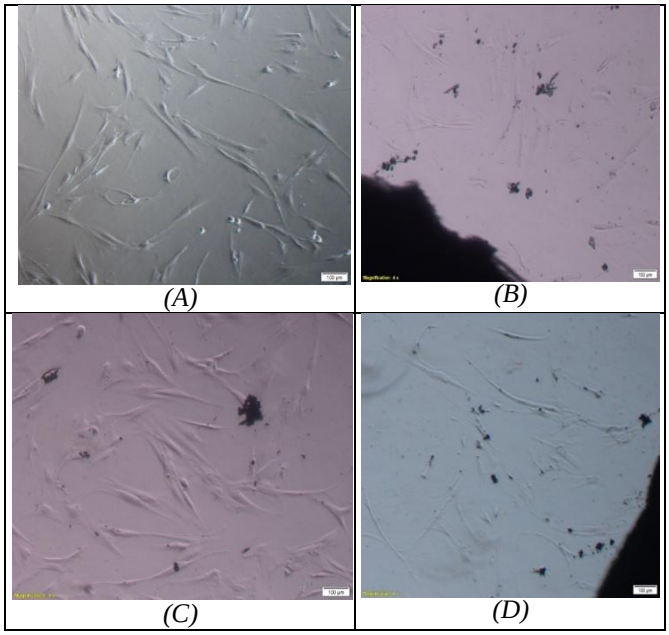


Figure 11: WPS9 cells response to BioRoot RCS is charecterised by leaching of material throughout (B, C, D, F, G). Reduced cell density is noted from the 5th day till the 7th day (E, G). Morphological changes in the cells can be noted from the 2nd day till the 7th day (C, D, F, H).

WPS9Periradicular fibroblasts cultured with Totalfill BC samples.

One dish containing WPS9periradicular fibroblasts in 10% FCS with no material sample was taken as a control *(A)*. WPS9 fibroblasts were very tolerant to the Totalfill BC samples throughout the 7 days. There were not many changes in the shape and morphology of the cells *(B, C)*. The material was displaced the next day after it was places leaving behind fragments near the original position. There was leaching of material from the sample on the fifth and the seventh day *(D)*. Except for a few cells which appeared to be stressed on the fifth and the seventh day, cells were relatively tolerant to the Totalfill BC samples *(F, G, H)*.



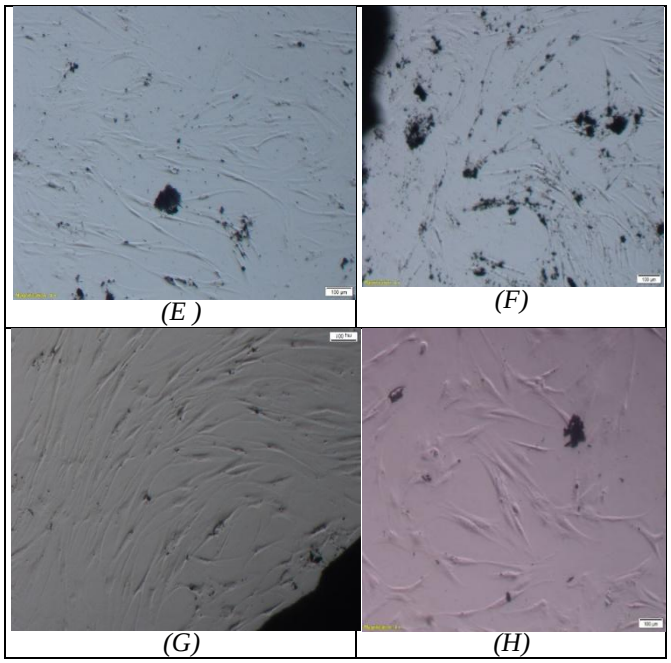


Figure 12: WPS9 cells in presence of TotalfilBcC sample do not show any drastic changes throughout. Cells appear to be normal till the 3rd day with little morphological changes (B, C, D, E). Cells are slightly stressed with negligible morphological changes on the 5th and the 7th day.

This experiment showed that tricalcium silicate cements induced more morphological changes in MM1 gingival fibroblast cell line. The greatest morphological changes were demonstrated in presence of BioRoot RCS then followed by Totalfill BC.

WPS9periradicular fibroblast also demonstrated some morphological changes though they were not as severe as that of MM1 gingival fibroblasts with respect to both the

materials. Excessive amount of cell death was noted in both the cell lines in case of AH+ sealer.

5. Discussion

In this study it was noted that the highest amount of cytotoxic effect of the sealer was noted with the AH Plus sealer as the cell death was almost 60% on the first day which increased to become 100% by the third day. This was in accordance with the study [15] where it was concluded that the bioceramic sealer was more biocompatible compared to the AH Plus sealer. The cytotoxic nature of the AH Plus sealer has been mentioned in various other studies as well, one study comparing the AH Plus sealer to an experimental calcium silicate cement using the MC3T3-E1 osteogenic cell line at 6 weekly intervals after immersion in a culture medium. It was observed that AH Plus happened to be more cytotoxic than the experimental calcium silicate cement [16]

The result of this study does not correspond to that another study where the cell adhesion to the material samples were evaluated using scanning electron microscopy. It showed that there was more cell adhesion to AH Plus sealer compared to Endosequence BC which is a calcium silicate-based root canal sealer [17]. Some of the early studies compared the cytotoxic effects of AH Plus and AH 26. Here it was found that the AH Plus sealer was bore biocompatible than AH 26 [18].A study looked at the effect of AH Plus, AH 26 and Zinc oxide eugenol-based root canal sealers on human gingival fibroblasts where AH Plus was found to be more biocompatible [19]. This can be due to the time frame in which the study was conducted and the material which was used to compare AH Plus. It was the time when the calcium silicate cements were not very popular in the market and the release of formaldehyde from AH 26 was widely recognised. Comparing these properties of AH 26 it was safe to say that AH Plus was the better option when it was first introduced in the market.

Not many studies are available which tell us in detail about the visual changes brought about by the Totalfill BC and Bioroot RCS. One such study which looks into this aspect where comparison of the biocompatibility between Totalfill BC, AH Plus and MTA Fillapex. It was noted that Totalfill BC happened to be the most biocompatible compared to the other two sealers [20] which corresponds with the result of our study.

6. Conclusion

Principle findings of this study include:

- The epoxy resin sealer AH+ was partially toxic to both gingival and periradicular fibroblasts.
- Tricalcium silicate-based root canal sealers alter the cell morphology of gingival fibroblasts.

7. Limitation of this Study

Only visual examination will not give a concreate picture of biocompatibility and cytotoxic profile of the materials. Hence more in vitro studies are required to reach a proper conclusion.

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