

CYTOTOXICITY OF ENDODONTIC SEALERS-A COMPARATIVE STUDY USING L-929 MOUSE SKIN FIBROBLAST CELL RESPONSE-AN EX-VIVO STUDY

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ABSTRACT

Aim: The objective of the present research was to evaluate and to compare the cytotoxicity of four commonly used endodontic sealers Apexit Plus, AH plus, Sankin, and Endofloss. The cytotoxicity was evaluated after setting of the sealers at different time intervals. **Materials and Methods:** Mouse skin fibroblasts L-929 was obtained from cell repository centre of national centre for cell science Pune, India. The cells were grown as monolayer cultures in Dulbeccos Modified eagle Medium (DMEM). Each of the test materials were mixed according to the manufactures instruction and was allowed to set. 0.1ml of each of the set sealers was placed in the petriplates in direct contact with the fibroblasts at 24 and 48 hrs intervals and evaluated for cytotoxicity. The percentage viability of the fibroblasts were calculated and evaluated statistically. **Results:** The statistical analysis revealed that Apexit Plus showed slight to moderate toxicity at 24 and 48 hrs, when compared with other sealers. Sankin showed maximum toxicity at all time intervals. **Conclusion:** All tested endodontic sealers demonstrated varying amount of cytotoxicity at different time intervals. Apexit Plus showed the least amount of cytotoxicity and Sankin showed the highest level of cytotoxicity.

INTRODUCTION

Endodontic therapy does not aim at rehabilitation of that particular tooth alone, but is concerned with the whole stomatognathic system. The materials, which are used during endodontic treatment should be non-toxic, friendly to the tissues of contact and also should not produce any systemic effect, in other words it should be bio-compatible. There are many types of root canal sealers in endodontics like zinc oxide eugenol, calcium hydroxide, glass ionomer, resins and silicon. The most desirable properties of a root canal sealer are its sealing ability and biocompatibility. Many studies have been carried out to check the biocompatibility of sealers using cell cultures and tissue implants[1]. Autian was the first to propose a structured approach at three levels while testing the material for cytotoxicity[2]. In this study we have made a comparative analysis of four commonly used endodontic sealers Apexit Plus, AH plus, Sankin, and Endofloss as there are very few studies making such a comparison.

MATERIAL AND METHODS

Ethical approval: Ethical approval was not taken as the study does not involve human beings.

Methodology:

Test Material: The four commonly used endodontic sealers used in the study are Apexit plus (calcium hydroxide based), AH plus (Resin based), Endofloss (Zinc oxide Eugenol based) and sankin (calcium

phosphate based). All the sealers were mixed according to the manufacturers instructions. Each set sealer was weighed and standardized. 0.1 ml of the set material was used for the evaluation of cytotoxicity.

Cell Culture: L 929 mouse skin fibroblasts (Passage number: 39) was obtained from cell repository centre of national centre for cell sciences Pune, India. The cells were grown as mono-layer culture in Dulbeccos Modified eagles medium(DMEM),Hi-Media laboratories limited,Mumbai,India)[2] with 2mM L-glutamine, Earles BSS adjusted to contain 1.5g/l sodium bicarbonate, 0.1mM non essential amino acids and 1mM sodium pyruvate (Himedia) supplemented with 10% fetal bovine serum (FBS, Himedia) and antibiotics (penicillin and streptomycin) in culture flasks (Himedia) at 37°C in an atmosphere of 5% CO₂.

According to the protocol given by Koulaoukidou et al (1998) 0.1ml of each of the set sealers were placed at the bottom of the petriplates[2] The set sealers were passed through UV light to prevent bacterial contamination. Each petriplate was covered with 2ml of cell suspensions at a final concentration of 4x10⁴ cells per petriplate.

All four sealer samples and respective controls without sealer were prepared in duplicate (a ,b) (Fig 1). All petriplates were incubated at 37⁰ under 5% CO₂ for 24 and 48 hrs. Dulbeccos medium was removed and the cells were detached by trypsinization. Cells were stained with trypan blue and viable cells were counted. Total cell count and viability percentage was calculated. The results were categorized according to the 4-point

cytotoxicity grading system by Hegde et al[3] According to this the cytotoxicity was rated based on the cell viability relative to control (Table 1).

Statistical Analysis: Percentage of cell viability was calculated from the following formula.

$$\% \text{ of cell viability} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100$$

RESULTS

The present study evaluated and compared the cytotoxicity of four commonly used root canal sealers Apexit Plus, AH Plus, Sankin and Endofloss, after setting and evaluated at 24 and 48 hours time intervals.

Direct exposure of Mouse skin fibroblasts to sealers for different time intervals revealed differential morphologic changes when viewed under inverted microscope at 200 X magnification. Normal untreated fibroblasts are generally spindle shaped in appearance with flattened and extended cellular processes that were attached to the petriplates, and the cell density was evenly distributed. In the experimental cultures, where the cell suspension was in direct contact with the different sealers, a cell-free zone adjacent to the sealers was seen (due to cytotoxic damage caused by sealer). All the sealers showed either mild to moderate cytotoxicity at different time periods.

After exposure of the fibroblasts to the test sealer samples, the fibroblasts retracted with residual cytoskeleton and with an increase in intercellular space. During viability counting when cell suspension was in direct contact with the sealers, most cells showed rounded appearance and were loosened from petriplates. Whereas in the control culture plates, it was observed that the cells were evenly distributed, and were attached to the petriplates .

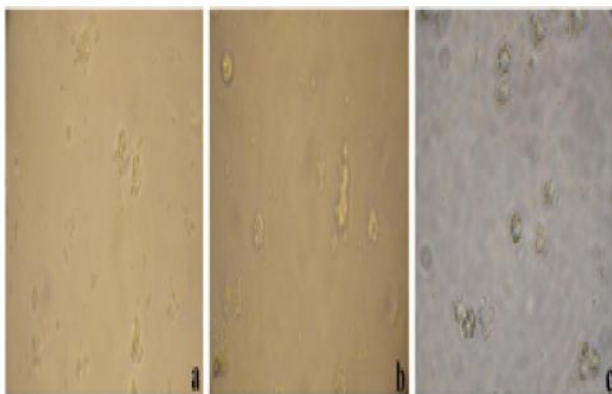


Fig 1: Cell morphology when in direct contact with the sealers: (a) and (b) L 929cell lysis; (c) rounded L929 cells loosened from the substrate in the presence of four Dental Sealers under 200X magnification

Endofloss which is a zinc-oxide eugenol based sealer was not completely hardened and showed some particles dissolved in the medium. Thus certain disintegrated small particles were found in the medium.

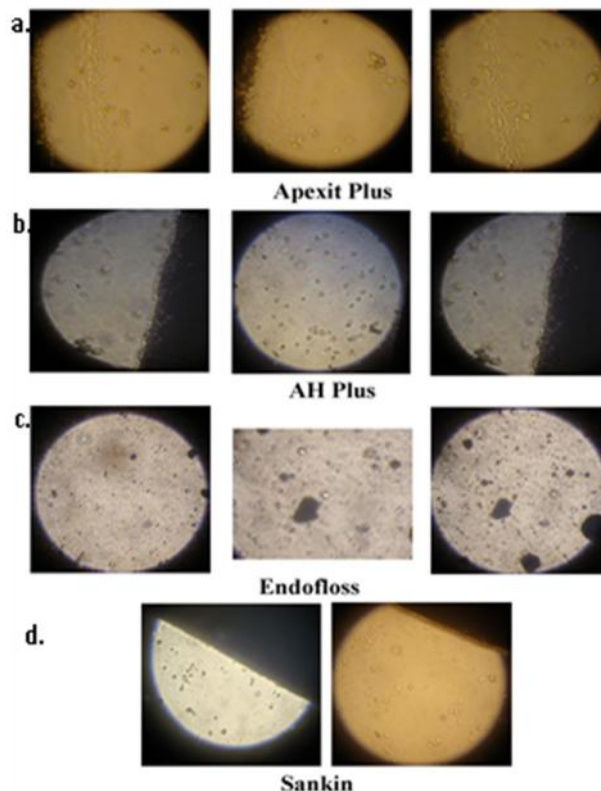
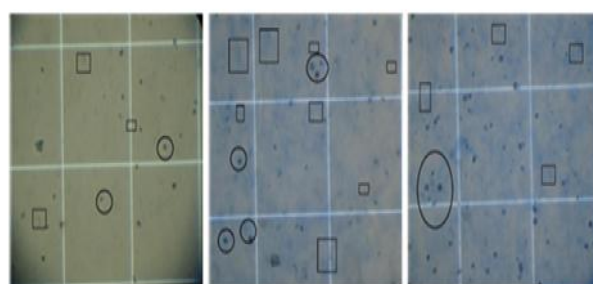


Fig 2: Effect of Endodontic Sealers on L929 cell lines under 100X magnification

After predetermined time period (24 and 48 hrs) the test materials and the medium were removed and trypsin was added to remove the cells from the bottom of the petriplate. The suspended cells were then mixed with trypan blue. The dead cells stained blue as they allowed the stain to enter their membrane, coloring their cytoplasm blue. The live cells excluded the stain, thus making the cells clear. Most of the cells could not exclude trypan blue, implicating the cell membrane damage and loss of cell viability during counting of viable cells using hemocytometer



Counting of cells in Neubauer hemocytometer, non viable (nv) cells [in square boxes] stained blue whereas viable (v) cells [in circles] were unstained

Fig 3: Trypan blue staining and counting of cells under 200X magnification.

The percentage of viable cells was determined (Table 2):The data obtained was statistically analyzed.All sealers showed cytotoxicity for L929 cells at all time periods with variation in toxicity.

At 24 hours period: AH Plus, Sankin, endofloss displayed no cell viability indicating strong cytotoxic activity at 24 hours of incubation where as Apexit Plus showed 46% cell viability. 48 hours: Apexit Plus ,AH Plus and

Endofloss showed a cell viability of 76%, 67% and 50% respectively indicating slight to moderately cytotoxic activity. At 24hrs the percentage cell viability of endodontic sealers was in the increasing order, Apexit Plus > AH Plus > Endofloss > Sankin. Apexit Plus showed least cytotoxicity compared to other sealers at 24 hrs time period, whereas the other sealers showed comparable cytotoxicity. After 48 hrs of incubation, Sankin showed maximum cytotoxicity, whereas there was no change in viability of cells in the presence of Apexit Plus and AH Plus.

Apexit Plus > AH Plus > Endofloss > Sankin

Table 1 : four point cytotoxicity grading system according to Hegde et al

CYTOTOXICITY	CELLVIABILITY
Non-cytotoxic	Greater than 90%
Slightly	60-90%
Moderately	30-59%
Strongly	Less than 30%

Table 2: cell viability count

Sealers	Percentage viability at different Time Periods Percentage of viable cells= (A/B) x 100*			
	24 hours	Cytotoxic	48hours	Cytotoxic
Apexit Plus	46 %	Moderate	76%	Slightly
AH Plus	0	Strongly	67%	Slightly
Sankin	0	Strongly	0	Strongly
Endofloss	0	Strongly	50%	Moderate

where, A= viable cells in the experimental petriplate, and B= viable cells in the control.

DISCUSSION

According to Carrote et al, the principle of endodontic therapy is to eliminate infection in the root canal and to fill three dimensionally the root canal space with bio-compatible, dimensionally stable, and chemically inert material so as to isolate any micro organisms that may remain within the root canal from nutrients in the tissue fluids [4] Bouillaguet stated that complete sealing of the root canal system is critical to prevent reinfection at periapical tissue [5] Guttapercha as a core obturating material is most popular and has always been used along with root canal sealers, irrespective of the technique of obturation. Root canal sealer helps to reduce the gap between core obturating material and the root canal wall, besides acting as lubricant. Endodontic sealers can come in direct contact with surrounding soft and hard tissues when extruded from the canal or the chemicals may leach through the canal and affect the periapical tissues [6] Thus it is imperative to evaluate the cytotoxicity of the commonly used endodontic sealers.

The cytotoxicity of a material can be evaluated either invitro or in vivo animal studies. In vitro studies utilize cell culture studies, which use either mouse fibroblast or human fibroblasts from periodontal ligament [7, 8] Mouse fibroblast L929 is commonly used as it resembles the connective tissues of human periodontal ligament fibroblasts. The biocompatibility of four commonly used endodontic sealers namely Apexit Plus(Calcium

hydroxide based), AH Plus (Resin Based) Sankin(calcium phosphate Based) And Endofloss(Zinc oxide eugenol based) was evaluated on mouse fibroblasts L929 at different time intervals of 24hrs and 48 hours.

The Mouse fibroblasts L929 in contact with most of the endodontic sealers showed maximum destruction at both 24 hours and 48 hours. Sankin, a calcium phosphate based endodontic sealer showed maximum cytotoxicity with no viable cells when compared to the control culture, which observed maximum viable cells. Similar results were corroborated by Koulaoudidou et al (1998) using direct counting to calculate the percentage viability[2] Studies have shown that the root canal sealers when inserted into the canal are in a freshly mixed incompletely polymerized state, and therefore during a relatively short period after clinical application local responses are provoked by partially reacted or unreacted components. Potentially cytotoxic materials are generally released during the setting period of the sealers.

According to the study conducted by Eldeniz et al, AH Plus significantly inhibited the growth of L929 cells and exerted a strong cytotoxic effect[9] Cohen inferred that the cytotoxicity was due to minute amounts of formaldehyde, amine and epoxy resin components present in the sealer [10]

According to the manufacturers, AH Plus is an improved formula of AH26 and the material no longer releases formaldehyde. However, amines are released, which are used to increase the polymerization in AH Plus and regarded as the primal reason for the initial toxicity [11]

Apexit Plus is a calcium hydroxide based sealer which showed the least amount of cytotoxicity in comparison to all sealers when tested in direct contact assay and in different time periods (24 & 48 hrs). It has shown cellular viability of (67%) which indicates its bio-compatible nature at 48 hours. This finding of our study is in agreement with the result of the studies conducted by Guigand et al, Schwarz et al, Desai and Chandler who showed similar favorable biocompatibility of calcium hydroxide sealers with more than 90% of cell viability in culture [12,14] As calcium hydroxide neutralizes the pH, the toxic effect subsides. Guigand et al suggested proliferation of cells due to liberation of calcium ions into the medium as free calcium ions have favorable effects on cell proliferation[13] However, the results of our study are inconsistent with the study conducted by Benjamin et al. and Camps and About who concluded that cell rupture and fragmentation were marked in cultures indicating the cytotoxicity potential of the calcium hydroxide based sealers[15, 8]

Zinc-oxide-eugenol content in Endofloss exhibited a toxic effect at all intervals (24 and 48 hrs) when in direct contact (50% cell viability). This result is in accordance with studies conducted by Zmener et al and Beagrie et al who reported zinc-oxide and eugenol have cytotoxic effect in several animal and human cell lines[11, 16] Similarly, Gerosa et al found that the cultures exposed at 24 hours, first week and second week test solutions of zinc oxide eugenol showed mild cytotoxicity. This was due to decrease in the release of eugenol [17] The results of the present study were in accordance to Kaplan et al who demonstrated that the zinc oxide eugenol

sealers are highly water soluble, releasing high amount of potentially cytotoxic substances[18]

Sankin is a calcium phosphate based endodontic sealer which showed variable results at different test intervals when in direct contact [0% cell viability]. Kangarloo et al evaluated the cytotoxic effect of four root canal sealers (AH plus, Sankin, Tubliseal EWT and Apexit) which were tested on fibroblast cells. The amount of Interleukin-6 (IL-6) released in response to the sealers was also evaluated by ELISA technique. Highest release of IL-6 level was found to be in Tubliseal EWT and Sankin groups when compared with AH plus and Apexit group. AH plus showed less cytotoxicity and induced less IL-6 release [19]

CONCLUSION

Within the limitation of the current study, all the sealers were slightly cytotoxic at their initial setting stages in comparison to the control group. Apexit plus was relatively biocompatible as compared to AH Plus, Sankin and Endofloss root canal sealers. The time intervals used in the present study were probably inadequate to predict the biological responses of extruded sealers that remain in contact with periapical tissues for decades. Use of human fibroblast instead of commercially available cell line may help to simulate human body and hence can give more predictable results. Further studies are necessary to determine the long term toxicity of sealer with more simulating clinical conditions.

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