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CYTOTOXICITY OF CALCIUM PHOSPHATE, EPIPHANY AND AH PLUS SEALERS- A COMPARATIVE EVALUATION BY MTT ASSAY

Dental Science

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ABSTRACT

Background: One of the principal requirements of an endodontic root canal sealer is that it should be non-cytotoxic, non-mutagenic and immunologically compatible with periapical tissues.
Aim: To determine the cytotoxic potential of Chitra-CPC for the first time and compare it to a commonly used sealer AH plus and a recent dual cure resin cement Epiphany, by a colorimetric MTT assay and thus explore the possibilities of these materials in Endodontics.
Materials and Methods: Cell cultures were obtained of mouse fibroblasts and subjected to the MTT colorimetric analysis with respect to exposure to the respective sealer and the percentage of viable cells was obtained for each group.
Results: The toxicity of the sealer tested in this study varied from Highest to lowest in the order: AH Plus> Epiphany>Chitra CPC. The differences were statistically significant between different sealers and at different time periods also.
Conclusion: Chitra-CPC, is relatively, a biocompatible sealer as compared to AH Plus and Epiphany Sealers

KEYWORDS:

INTRODUCTION

Successful endodontic treatment depends on thorough debridement of the root canal(s) followed by three dimensional obturation. The objective of the obturation, in the endodontic treatment is to eliminate all the pathways between the periodontium and the root canal by sealing the root canal completely with a condensed, dimensionally stable, bio-inert filling material[1]. Gutta percha cones are the solid part of the filling, whereas sealers help to fill in all the remaining empty spaces, foraminae as well as accessory and lateral canals thus providing a three-dimensional filling[2,3].

Over extrusion of the sealer or the elutable substances from the sealer can enter the periapical tissues and cause inflammation, degeneration of the tissues lying underneath the sealer, delay wound healing and cause pain resulting in the failure of otherwise well done root canal treatment.[4,5,6] Hence one of the principal requirements of an endodontic root canal sealer is that it should be non-cytotoxic, non-mutagenic and immunologically compatible with periapical tissues[1, 3]. Root canal sealers and their elutable components, therefore, need to be critically evaluated for their cytotoxicity and genotoxicity prior to their general clinical use. There are number of ways to test the in vitro cytotoxicity of a material,[7] and MTT assay is a sensitive, rapid, well accepted and most widely used method.

Most of the sealers used in endodontics have some biocompatibility issues. Since last three decades, Calcium Phosphate cements (CPC) have emerged as new generation materials and highly valued for their biocompatibility.[10] Research is still on to develop CPC formulations for various clinical applications in dentistry including root canal sealers.(r) In an indigenous venture, scientists at Shree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram have developed a new formulation of CPC named Chitra-CPC which has enhanced viscous and cohesive properties than conventional CPC making it suitable to be used as sealer.[8-10]

The present study is aimed at determining the cytotoxic potential of Chitra-CPC for the first time and compare it to a commonly used sealer AH plus and a recent dual cure resin cement Epiphany, by a colorimetric MTT assay and thus explore the possibilities of these materials in Endodontics.

The objectives of this study are:

1. To evaluate the cytotoxic effects of three different root canal sealers: Epiphany Sealer(Pentron USA), AH-plus sealer (Dentsply) and an experimental calcium phosphate sealer(Chitra-CPC) on mouse fibroblasts (L929) using MTT assay, during different time periods of 0 h,24 h and 7 days after mixing.
2. To compare cytotoxicity among above mentioned materials at different time periods after mixing.
3. To observe mouse fibroblasts (L929) under inverted phase contrast microscope for changes in the cell morphology after contacts with elutes of these sealers.

MATERIALS AND METHODS

The sealers tested in the study are Chitra-CPC (SCTIMST, India), Epiphany (Pentron, USA) and AH-Plus (Dentsply, Germany). The sealers alongwith their composition are listed in Table-1

Table-1

	Sealer	Manufacturer	Composition as per manufacturer
1	Chitra-CPC (Calcium Phosphate Based Sealer)	Biomedical Technology Wing, SCTIMST, Thiruvananthapuram, India	Powder:- Tetracalcium Phosphate and Dicalcium Phosphate Dihydrate particles of size 50 µm mixed in equimolar ratio. Liquid:- Distilled water with Na2HPO4 as the setting acceleiator in an optimized concentration of 0.2 M

2	Epiphany (Dual-cure resin sealer)	Pentron Clinical Technologies, LLC, Wallingford, CT,USA	Mixture of Urethane dimethacrylate (UDMA), Poly (ethylene glycol) dimethacrylate (PEGDMA), Ethoxylated bisphenol-A dimethacrylate (EBPADMA), Bis-GMA resins, silane treated barium borosilicate glasses, barium sulphate, silica, calcium hydroxide, Bismithoxychloride with amines, Peroxide photoinitiator, stabilizers and Pigments.
3	AH-Plus	Dentsply, De Trey Konstanz, Germany	Paste-A:- Epoxyresin, calcium tungstate, Zirconium oxide, erosil, Iron oxide pigments. Paste-B:- Adamantane amine, Calcium tungstate, Zirconium Oxide,, N,N-dibenzoyl-5-oxanonane diamine-1, I-TCD-diamine, Silicone oil

Methods Of study:-
The experiment was divided as under:-

Growing cell cultures:-
L929 mouse fibroblasts (ATCC, USA) were cultured in the 25 cm^2 culture flasks containing Eagle's Minimum Essential Medium (MEM; SIGMA USA) supplemented with 10% fetal bovine serum (SIGMA, USA), and containing Penicillin-G 100,000 IU/ml; Streptomycin 100,000 ug/ml and 30mg/ml L- glutamine, in an atmosphere of 5% CO_2/95% air mixture at 37 °C. After thorough rinsing and trypsination, cells were seeded at a density of 5×10^3 cells per well in 96 well culture plates (Nunc,Denmark) and incubated for 24 h in 5% CO_2/ 95% air at 37 °C, to get sub confluent monolayers of cells.

Sample preparation and Elution:
All the three sealers were mixed aseptically inside laminar flow hood (Clean air systems & Devices; USA) according to Manufacturer's instructions. After mixing, samples of each sealer were dispensed as almost round disc like specimens into preweighed autoclaved glass vials, using a tuberculin syringe. The premeasured volume of each sealer is dispensed into each vial and these vials are again weighed. The vials with 100mg ± 2mg weight samples are taken for the study. Three such samples per material per time period are taken for the study. So, 9 samples per material and total 27 samples are taken for study. The samples are divided into three groups as[Plate III]:-

Group I (0 h group)- three samples/sealer and eluted immediately after mixing and dispensing.

Group II (24 h group)- three samples/sealer and eluted 24 h after mixing.

Group III (7 day group)-three samples/sealer and eluted 7 days after mixing.

Following 0 h, 24 h and 7 days after mixing, samples are eluted as per **ISO-10993-12, 1996** by adding 1 ml of media containing serum in each vial with the help of a micropipette (Pipetman; Gilson; France.) at 37 ±2 °C for 24±1 h at an extraction ratio of 0.1g / ml.

Addition of elute or extract to the cells:-
Invitro cytotoxicity test using extract on cells was done based on the **ISO 10993-5, 1999**, guidelines. At the end of elution, 100 microlitres of this test elute is added per well of already cultured L929 mouse fibroblasts in 96 well culture plates. This elute is added to six cell wells per material per time period. Six cell wells per material with 100 microlitres of 130 mg% Phenol (SIGMA, Chemicals, USA) acts as positive control and six cell wells without any addition serves as negative control. Then these cells are incubated for 48 h in 5% CO_2/ 95% air mixture at 37 °C in an incubator (Nuaire, USA).

MTT assay:-
The MTT assay, first described by Mosmann[10] in 1983 focuses on the capacity of mitochondrial dehydrogenase enzyme in **living cells** to convert the yellow water soluble tetrazolium salt 3,4(4,5-dimethylthiazole-2-YL)-2,5-diphenyltetrazolium bromide (MTT, SIGMA,Chemicals,USA) into dark blue formazen crystals. These water insoluble crystals are stored in the cytoplasm of living test cells, as the cells are largely impermeable to them. The number of surviving cells is thus, directly proportional to the level of the formazan product formed. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals which are solubilized. The color can then be quantified using a simple colorimetric assay. The results can be read on a multiwell scanning spectrophotometer (ELISA reader).

After 48h of incubation period of cells in the presence of test elutes, elutes and the media are removed from the culture wells. Then 200 microlitres of MTT working solution (0.5 mg of MTT per ml serum free media) is added to each well .The culture plates are wrapped with aluminum foil and incubated overnight in a humified atmosphere at 37 °C. After this, MTT solution is removed from the culture plates and 200microlitres of propanol is added using multichannel pipette (Pipetman, France) and plates are incubated at 37 °C for 20 minutes in a shaker incubator (Environ Shaker, Lab-Line, USA) for Solubilisation of crystals. After that immediately the spectrophotometric absorbance is read at 570 nm, with reagent alone as blank under ELISA reader (Biotech EL 311S, USA). The experiment was repeated twice and we got the same results each time.

The percentage of the viable cells is calculated from Optical density values, using the formula –

$$\text{Percentage of viable cells} = \frac{\text{Optical density of test well}}{\text{Optical density of negative control.}} \times 100.$$

Then the Mean and standard deviation of these percentage values (six values per material per time period) for each material and time periods is calculated. These values are analyzed statistically using parametric test like one way ANOVA to know the statistical significant differences between the materials and between the time periods and these materials are further compared in each group for significance using Duncan's Multiple range test.

RESULTS
The mean percentage of viable cells for different materials tested along with their standard deviations (SD), F value and P value at different time periods (0 hr, 24 hrs & 7 days) are given in the Table 2.

Table 2. Analysis of variance (ANOVA*)of percentage of viable cells comparing Materials (0 hr, 24 hr, 7 Days) after mixing

Table 2

Observations Values of Materials- 0 Hour (Immediately) after mixing				
Materials	**Mean Percentages**	**+ SD**	**F value**	**P value**
Chitra – CPC	25.91[c]	0.67	31.690	< 0.001
AH Plus	23.90[b]	1.12		
Epiphany	21.61[a]	0.96		
Observations Values of Materials- 24 Hours after mixing				
Materials	**Mean Percentages**	**+ SD**	**F value**	**P value**
Chitra – CPC	97.72[c]	4.67	1359.667	< 0.001
AH Plus	19.96[a]	0.73		
Epiphany	28.57[b]	1.30 Observations Values of Materials- 7 Days after mixing		
Materials	**Mean Percentages**	**+ SD**	**F value**	**P value**
Chitra – CPC	99.73[c]	3.53	1560.956	< 0.001
AH Plus	21.64[a]	1.17		
Epiphany	35.33[b]	2.49 a, b, c – Means with same superscript do not differ each other (Duncan's Multiple Range Test) **		

The cytotoxicity of all the three sealers differ significantly from each other at 0 hr group, 24 hr group and 7 day group ($p< 0.001$)*.

At 0 hr, Epiphany sealer is significantly more cytotoxic than AH plus which is more cytotoxic than Chitra –CPC **. All the three materials tested have severe cytotoxicity at 0 hr, as more than 70% of the cells are non-viable.

At 24 hr, AH Plus sealer is significantly more cytotoxic than Epiphany sealer which is significantly more cytotoxic than Chitra –CPC**. Two of the three materials tested i.e. AH plus and Epiphany sealers have severe cytotoxicity as more than 70% of the cells are non-viable. But Chitra –CPC has become non cytotoxic as the 97.72 % cells are viable. At 7 days,

AH Plus sealer is significantly more cytotoxic than Epiphany sealer which is significantly more cytotoxic than Chitra –CPC**. AH Plus still showed severe cytotoxicity as more than 70% of the cells are non-viable. Epiphany sealers showed moderate cytotoxicity as percentage of viable cells is more than 30% (35.33%). But Chitra –CPC has become non cytotoxic as the 99.73% cells are viable.

Comparing time periods among same materials:-

The mean percentage of viable cells for Chitra-CPC, AH –plus and Epiphany sealers along with their standard deviations, F value and P value for 0 h, 24 h and 7day groups are given in the Table 3.

Table 3. Analysis of variance (ANOVA*)of percentage of viable cells comparing Time periods for Chitra-CPC, AH Plus, Epiphany sealers

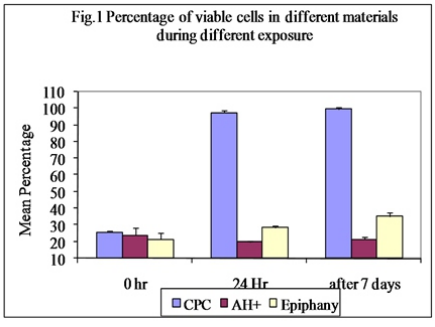
Percentage of viable cells for Chitra-CPC sealer				
Time Periods	**Mean Percentages**	**+ SD**	**F value**	**P value**
0 Hour	25.91[a]	0.67	915.115	< 0.001
24 Hours	97.72[b]	4.67		
7 Days	99.73[b]	3.53		
Percentage of viable cells for AH Plus sealer				
Time	**Mean Percentages**	**+ SD**	**F value**	**P value**
0 Hour	23.90[c]	1.12	22.364	< 0.001
24 Hours	19.96[a]	0.73		
7 Days	21.64[b]	1.17 Percentage of viable cells for Epiphany sealer		
Time Period	**Mean Percentage**	**+ SD**	**F value**	**P value**
0 Hour	21.61[a]	0.96	96.429	< 0.001
24 Hours	28.57[b]	1.30		
7 Days	35.33[c]	2.49 a, b, c – Means with same superscript do not differ each other (Duncan's Multiple Range Test		

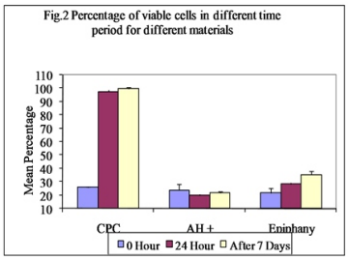
At 0 hr group cytotoxicity of Chitra-CPC is severe (25.91% viable cells only) which is significantly more from cytotoxicity for 24 hr and 7 day groups**.

For all the time periods the cytotoxicity for AH Plus is severe as Mean percentages of viable cells is 23.9, 19.96 and 21.96 for 0 hr, 24 hr and 7 day groups respectively, which is less than 70%.

For the 0 h and 24 h groups, the cytotoxicity of Epiphany sealer is severe (21.61% and 28.57 %viable cells only) which is significantly more from cytotoxicity for 7 day group**(moderate cytotoxicity-35.33% viable cells)

The bar diagrams showing the comparison of mean percentage of cells plotted for different materials and different time periods are:-





DISCUSSION

Calcium Phosphate cements are the emerging class of bone substitute materials due to the similarity of the set cement in chemical composition to that of the bone material, hydroxyapatite.The Chitra-CPC is a fully injectable calcium phosphate cement that was developed and patented by biomedical wing of SCTIMST for orthopaedic and dental applications. [8]

Chitra –CPC has a setting time of 15-20 minutes, shows excellent handling characteristics and adequate working time which enables it to be used as a sealer for obturating root canals. It has enhanced viscous and cohesive properties than conventional CPC.It can be mixed in various consistencies, from mouldable putty to injectable paste. Besides this, it has a neutral pH during setting, is highly adaptable and adheres to root canal surface. It is also dimensionally stable,easy to handle and has the property of osteotransductivity.

Various tests for optimising its physical, chemical and manipulation properties have already been conducted by Komath et al.9. A battery of tests to evaluate the material safety and efficacy has been done by Fernandez et al 10 in various animals as per the ISO 10993 criteria and Chitra-CPC has qualified(in mice) for systemic response,(in rabbits) for skin response & pyrogenicity,(in guinea pigs) for allergic skin response, muscle and bone implantation tests (in rabbits). Recently George et al[11] demonstrated that Chitra-CPC is kind to periapical tissues when used as root canal sealer in porcine model by endodontic usage test. The present study is done to evaluate the cytotoxicity of Chitra-CPC for the first time by MTT assay on mouse fibroblasts L-929.

Using cell lines is a common method of invitro testing of sealers, which allows for reproducible results that can be controlled in a laboratory setting. In vitro testing also allows for the comparison between several materials using the same cells under the same conditions.[12] L-929 mouse fibroblasts is one of the most widely used cell line for invitro assays. It is an ATCC certified and established cell line which is readily available and gives reproducible results, hence used in the present study for cytotoxicity evaluation.[13,14]

In this study, MTT assay was used. The advantage of this method is its simplicity, rapidity, precision and it does not require radioisotopes. It is a well established calorimetric assay for quantitative measurement of metabolically active cells. It focuses on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water soluble tetrazolium salt 3,4(4,5-dimethylthiazole-2-YL)-2,5-diphenyltetrazolium bromide into dark blue formazan crystals. Since dead cells are unable to produce coloured formazan crystals, this assay can be used to distinguish viable cells from dead cells. The number of surviving cells is thus, directly proportional to the level of the formazan product formed.[15,16]

In this experiment, the extract of sealer was prepared as per ISO-10993-5, 1999 guidelines.[17] The cytotoxicity of sealers was checked immediately after mixing, 24 hr after the mixing and 7 days after mixing the sealer. In the present study, cytotoxicity of two new root canal sealers Chitra-CPC and Epiphany has been tested and compared with that of widely used AH plus sealer.

The Chitra-CPC is having severe cytotoxicity (only 25.9% of cells are viable) immediately after mixing, which completely subsided at 24hrs (97% of viable cells) and there after reaches total normalcy (99.73% viable cells) after 7 days. The short term cytotoxicity of this sealer has been attributed to high solubility of the sealer in water which results in large amount of release of ca^{++} & P^{+++} ions into solutions as advocated by the manufacturers.[8] This increase of Ca^{++} ions in the initial phases changes the ca^{++} balance in & around the cells and thus alters its

membrane permeability, metabolic function and results in the destruction of the cells.

Once the sealer is set, this release of ca^{++} ions is negligible, which does not affect the cells & the cells can normally grow & proliferate in its presence. So, this sealer can be considered biocompatible after setting. The results of this study are in agreement with the few invitro and in vivo studies conducted elsewhere using somewhat similar formulations of calcium phosphate cements [19,18].The cytotoxicity of 0 h group was significantly different from 24 h and 7 days groups, at which time periods, it was totally non-toxic.

The Epiphany sealer showed severe cytotoxicity at 0 h (21.6% viable cells) and 24hrs (28.57% viable cells) which is reduced to become moderate after 7 days (35.33%).This high toxicity can be due to presence of un polymerized hydrophilic monomers such as HEMA,which shows diffusibilty in the surrounding tissues and create an un-cured surface layer for extended time periods.[20] HEMA can also suppress cellular growth and cell cycle progression.[21] The toxicity of Epiphany has been also revealed in previous studies.[12,20,22]

The AH plus sealer has shown severe cytotoxicity at 0 h (23.9% viable cells), 24 hrs (19.95% viable cells) and 7 days (21.64% viable cells). As suggested by many [23, 24] the epoxy component of AH plus and the various amines in the composition of material, may be related to toxicity of these materials. The results of our study are in agreement with many other authors, who have found AH plus to be cytotoxic. [5,18,25, 26,]

The toxicity of the sealer tested in this study varied from Highest to lowest in the order: AH Plus> Epiphany>Chitra CPC. The differences were statistically significant between different sealers and at different time periods also.

Since sealers were tested by an invitro cell culture method, results may not be directly comparable with the in vivo conditions where all healing parameters are functional. But this study gave us a firsthand knowledge about the cytotoxicity of a new sealer Chitra-CPC and also provided valuable insight into the cytotoxicity potential of other two sealers tested. More & more in vivo studies and studies for longer time periods are needed in the future before we come to a conclusion about the definite biocompatibility of these materials.

CONCLUSION

Within limitations of our current study, it is concluded that Chitra-CPC, is relatively, a biocompatible sealer as compared to AH Plus and Epiphany Sealers, but more and more studies regarding their in vivo biocompatibility, sealing ability and other properties has to be conducted so as to search for the development of an ideal root canal sealer as proposed by Grossman.

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