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## Cytotoxicity of Newer Retrograde Filling Material on Human Gingival Fibroblasts: An Invitro Study.

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### ABSTRACT

To evaluate the cytotoxicity of newer retrograde filling material( ie: biodentine , mineral trioxide aggregate angelus and glass ionomer cement) on human gingival fibroblasts cell line by means of the sulforhodamine b assay, a new method in toxicity screening of dental materials. 3 samples each of biodentine (Septodont) , mineral trioxide aggregate angelus and glass ionomer cement (Fuji type IX Gold) were prepared for the study and set at 37C in 100% relative humidity for one day . The set materials were immersed in dulbecco modified eagle culture medium for 24 hrs. Fibroblasts cultured in dulbecco modified eagle medium were used as a control group.The test materials extracts were then separated and then tested in culture wells in close proximity to growing cell culture and incubated for 24 hrs . Cytotoxicity/ Survival fraction was estimated by Sulforhodamine B assay, in reference to controls. Cells exposed to extracts from MTA angelus and biodentine showed the highest survival fraction % after 24 hrs , whereas cells exposed to glass-ionomer cement type IX gold extracts displayed the lowest survival fraction %. The degree of cytotoxicity in ascending order was biodentine, mta angelus & glass-ionomer cement type IX in the cell line tested.

**Keywords:** Biocompatibility, biodentine, cytotoxicity, Sulforhodamine B assay ,glass ionomer cement , human gingival fibroblast.

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## INTRODUCTION

Success of endodontic surgery depends upon the sealing ability of retrograde filling material. This is made possible by the osteoproliferative effect of the retrograde filling material that would promote bone formation in close contact with the tissues. Various new calcium - silicate based retrograde filling materials have been introduced recently [1].

Mineral trioxide aggregate (MTA) has numerous clinical applications. Since its first description in the dental literature, it has been used as pulp capping material, root-end filling material and root or furcal perforation repair material [1].

Biodentine (Septodont, Saint-Maur-des-Fossés, France) was introduced in 2011. It has potential benefits but not the drawbacks of other bioceramic cements. It contains tricalcium silicate, calcium carbonate, and zirconium oxide and a water-based liquid-containing calcium chloride as the setting accelerator and a water-reducing agent. It is a fast-setting calcium silicate-based restorative material recommended for use as a dentin substitute that can be used both as a coronal restoration material, for perforation repair and as a pulp-capping material in direct contact with the pulp [3].

Cytotoxicity of a root filling material has a significant influence on the viability of periradicular cells and cause cell death by apoptosis or necrosis when used in perforation repair, or as a retrograde filling. To promote healing and restoration of the function of the tooth, dental materials should either stimulate repair or be biologically neutral. Therefore, it is essential to avoid dental materials that are toxic to the pulpal and periapical tissues that might compromise the clinical outcome [3,4].

Studies with cell cultures may offer a significant tool to improve our knowledge of possible toxic effects of materials and for predicting these effects on humans. As an alternative to *in-vivo* experiments, *in vitro* tests are simple to perform, repeatable, cost-effective, relevant and suitable in most conditions [1].

Cytotoxicity screening tests with cell cultures offer an excellent tool to improve our knowledge of possible toxic effects of retrograde filling materials and for predicting these effects on humans.

Anti-proliferative tests are more effective and reliable in determining the cytotoxicity of the root filling material, since it is a direct measure of the proliferation of the growing cells in direct contact with the root filling material [8].

The biocompatibility of MTA, biodentine and glass ionomer cement being used as a root end filling material has been evaluated both *in vitro* and *in vivo* studies. According to an *in vitro* study conducted by Bonson et al, MTA can stimulate periodontal ligament fibroblasts to display osteogenic phenotype and produce osteonectin, osteopontin, and osteonidogen [7].

However, till date, Biodentine has not reported its biocompatibility based on its assessment of anti-proliferative effect on fibroblasts cells.

### Aim and Objectives of the Study

The aim of the study was to evaluate the cytotoxicity of three retrograde filling material (Mineral trioxide aggregate, Biodentine and Glass-ionomer cement) against human gingival fibroblasts cell lines.

## MATERIALS AND METHODS

### Material used

3 test materials ie: Biodentine (Septodont), Mineral trioxide aggregate angelus white (Angelus) and Glass ionomer cement (Fuji type IX gold) were used for the study.

Human gingival fibroblasts cells were obtained from freshly prepared cell lines from human gingival tissue of healthy patients who underwent extraction in the dept of oral and maxillofacial surgery.

**Sample Preparation:**

The samples were divided into four groups :

**Group I – Control Group :** Human gingival fibroblasts

**Group II -Test Group 1:** Biodentine+ human gingival fibroblasts (3 samples)

**Group III -Test Group 2:** Mineral trioxide aggregate + human gingival fibroblasts (3 samples)

**Group IV -Test Group 3:** Glass ionomer cement (type IX) + human gingival fibroblasts (3 samples)

**Methodology**

The study was done in the central research laboratory of A.B. Shetty Memorial Institute Of Dental Sciences, Mangalore, Karnataka, India.

**Cell Culture**

Cells were grown as monolayer cultures in T- 75 flasks with culture medium containing dulbecco's modified eagle medium ( DMEM, Gibco, Glassglow, UK) added with 10 % fetal bovine serum + 100 mg/ml of streptomycin + 100IU / ml of penicillin. They were sub cultured twice / week at 37°C , 5% CO<sub>2</sub> in air , 100% humidity (passage no - 18-21).

This was followed by trypsinisation where the adherent cells are detached with 2-3ml of 0.05% trypsin ( Gibco, 1: 250) followed by incubation for 2 to 5 min at 37° C . The cells were then plated in the cell plate( 30,0000 cells /well) followed by incubation for 24hrs for growth .

**Preparation of Cement Elutes**

3 samples each of biodentine, mineral trioxide aggregate angelus and glass ionomer cement shaped with 3-mm thick molds with a diameter of 3 mm, according to the manufacturer's instructions under aseptic condition and set at 37c in 100% relative humidity for one day . After setting, the disks were exposed to ultraviolet light for 20 minutes on each surface to ensure sterility and transferred into 24-well tissue culture plates immersed in 1 ml DMEM per well for 24 hrs. DMEM without the materials incubated for 24 hours was used as control.

The test materials extracts were then separated and tested in Insert wells in close proximity to growing cell culture for 24 hrs. Cell number estimated by sulforhodamine b assay.

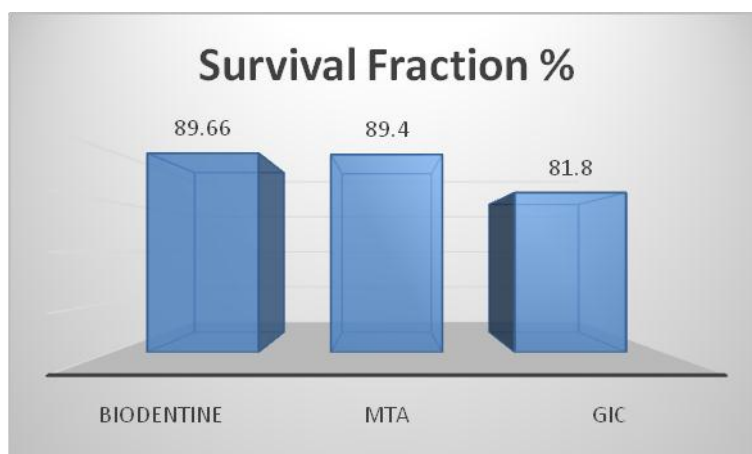
**Sulforhodamine B assay**

Culture medium is aspirated before fixation and 300 ml of 10% cold tri chloroacetic acid is added to wells. After microplates are left for 30 min at 4 C, they are washed 5 times in deionized water. Then left to dry at room temperature for 24hrs. 300 ml 0.4% (w/v) sulforhodamine B (Sigma) in 1% acetic acid solution are added to each well (left at room temp for 20 min) . SRB is removed and plates are washed five times with 1% acetic acid before air-drying. Bound SRB is solubilised with 600 ml 10 mm unbuffered tris-base solution (E. Merck, Darmstadt, Germany), plates are left on plate shaker for at least 10 min. Absorbance is read at 492 to 510 nm range .Experiments are performed in triplicates. Optical density will be determined by spectrophotometric analysis and expressed as survival fraction (sf), in which  $sf = ODx/ODc$  (ODx -optical density of the test wells and ODc - optical density of the control where empty insert wells placed ). Kruskal wallis test and Mannwhitney test were used to evaluate the statistical significance of the results.

**RESULTS**

MTA angelus and biodentine exhibited lesser antiproliferative effect, whereas glass ionomer cement (type ix) was more toxic. Overall, glass ionomer cement (type ix) inhibited cell numbers by 8% when compared to mta angelus and biodentine after 24hrs of exposure. No statistical significant difference was found between mineral trioxide aggregate and biodentine in the cell line at 24-h exposure. Glass ionomer cement (type ix) cytotoxicity was highest after 24 hrs indicating a short and time-independent effect. The effect of biodentine was more noticeable with highest survival fraction % in a 24hr period (graph 1). There was a significant inhibition of cell number that was evident even at 12 h, with a clear evidence of cellular death in the reverse microscope. Viability of the remaining cells was significantly impaired because cell numbers continued to decrease throughout the duration of the experiment.

**Graph I: Survival Fraction % after 24hrs**



Based on Kruskal wallis test (Table 1), there was a significant difference between the first, second and the third groups. Based on Mannwhitney test (Table 2), multiple comparisons were made and there was a significant difference between the MTA(Test group 2) and the GIC(Test group 3), the biodentine (Test group 1) and the GIC(Test group 3) but there was no statistical significance between MTA angelus and biodentine during the 24hr experimental period.

**Table 1: Kruskal Wallis Test: Comparing The Three Groups**

	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Kruskal wallis test value	p
				Lower Bound	Upper Bound		
Survival Fraction (%) At 24 hrs	3	89.66	.45	88.55	90.78	7.081	.029
Biodentin	3	89.41	1.47	85.76	93.05		
MTA	3	81.81	2.41	75.82	87.79		
GI	3	81.81	2.41	75.82	87.79		
Total	9	86.96	4.12	83.79	90.13		

**Table 2: Subgroup Analysis: Mannwhitney Test**

			Mean Difference	Std. Error	p	
Survival Fraction (%) At 24 hrs	Biodentin	MTA	.258	1.346	.834	
		GI	7.858	1.346	.023	sig
	MTA	GI	7.599	1.346	.002	sig

## DISCUSSION

Materials used in endodontics should preferably be biocompatible when they are placed in direct contact with living tissue such as in pulp capping, perforation repair, or when used as a retrograde filling.[9] Survival fraction depended on the type of material, culture medium and incubation time that the cells were exposed to [1]. In the present study, human gingival fibroblasts cells were obtained from previously prepared fresh cell lines from human gingival tissue of healthy patients who underwent extraction in the dept of oral and maxillofacial surgery.

Biodentine, as a retrograde filling material has excellent sealing ability due to its biomimetic mineralization quality. Therefore it is crucial to predict its possible cytotoxic effects on human gingival fibroblasts cells [3].

MTA has been extensively studied and recognized as a bioactive and biocompatible material since it has been satisfactorily used as the gold standard bioceramic cement.[4] Glass ionomers cements because of their adhesiveness and release of fluoride, have also gained popularity as the filling material in the treatment of cervical resorptions. Fuji IX, because of its fine grain powder and polyacrylic acid content , it is used as a conventional GIC that has a relatively high viscosity .

The characteristic of a short setting time of fuji IX GIC is similar to biodentine and may be an advantage in certain clinical situations such as perforation repair [10,11]. Fuji IX GIC solubility to oral fluids is low and it is popularly known for its rapid set due to its relative resistant to early moisture.[9] In the present study, the biocompatibility of MTA angelus and fuji IX GIC was evaluated in comparison with biodentine.

Several methods and strategies are available for cytotoxicity testing of materials that are simple, rapid, reproducible and inexpensive. The sulforhodamine b (SRB) assay was developed by shehan and colleagues to measure drug-induced cytotoxicity and cell proliferation for large-scale drug-screening applications.[12] It is used for cell density determination, based on the measurement of cellular protein content. The method described here has been optimized for the toxicity screening of compounds to adherent cells in a 24-well format.[8] Its principle is based on the ability of the protein dye sulforhodamine b to bind electrostatically and ph dependent on protein basic amino acid residues of trichloroacetic acid-fixed cells.[12] The method not only also requires simple equipment and inexpensive reagents but also allows a large number of samples to be tested within a few days. The srb assay possesses a colorimetric end point and is nondestructive and indefinitely stable. The srb assay is therefore an efficient and highly cost-effective method for cytotoxicity screening [13].

The cytotoxicity of the degradation products and elution substances from endodontic cements has to be taken into account , since it might gain access to periodontal tissues in numerous conditions, affecting the healing process [14]. Therefore, extracts of various concentrations derived from biodentine, mta and gic were examined for cytotoxicity. Here toxic elements of the retrofilling material leach into the surrounding fluids in the bony crypt , hence the extracts simulate the postsurgical root-end environment when placed in the culture medium [15].

Cements extracts from GIC fuji IX caused significantly more cell death than extracts from biodentine & MTA angelus after culture for 1 day. In order to determine the cell interactions with the materials, the surface topography of biomaterials is an important factor . Both biodentine and MTA shows crystalline, uneven surface topography, whereas GIC surfaces appeared smooth.[3] In general, a relatively smooth surface topography favors cell adhesion and growth. Even though there was increased cellular adhesion on the surface of GIC there was simultaneous cell death taking place . This could be likely due to the poor initial spreading of fibroblasts in a 24hr exposure period on the GIC type IX compared with biodentine & MTA angelus caused by leaching of toxic degradation substances such as aluminum and/or iron ions present in GIC extracts that adversely affect cell interactions with the material [16,17].

Based on a study conducted by Tatjana kanjevac et al, among the 8 tested GIC products, GIC fuji type IX & fuji plus showed the maximum release of fluoride and simultaneous inhibiting of cell growth, proliferation, mitochondrial activity and protein synthesis as a result , causing increased necrosis and cell death [18,19].

Therefore, fluoride release was in direct correlation with cytotoxic activity of GICs on human gingival fibroblasts cells & along with other toxic degradation products, excess fluoride release is also considered toxic.

### CONCLUSION

Based on the experimental method used in the present study, biodentine proved to be the most biocompatible materials in a 24hr experimental period. Cells exposed to extracts from MTA Angelus and biodentine showed the highest survival fraction % after 24 hrs, whereas cells exposed to Glass-ionomer cement type IX Gold extracts displayed the lowest survival fraction %. Biodentine caused gingival fibroblast reaction similar to that by MTA. In contrast, GIC showed significantly higher cytotoxicity levels than biodentine and MTA angelus.

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