In vitro Biocompatibility Tests of Glass Ionomer Cements Impregnated with Collagen or Bioactive glass to Fibroblasts

Subbarao C * / Neelakantan P ** / Subbarao CV ***

Aim and Design: *To evaluate the biocompatibility of glass ionomer cement (GIC) impregnated with collagen or bioactive glass to BHK-21 fibroblasts in vitro. Mineral Trioxide Aggregate was used as the standard for comparison. Human maxillary central incisors (n=70) were instrumented with a rotary NiTi system and filled. Following resection of the apical 3mm, root end cavities were prepared and restored with conventional GIC (group 1) or GIC with 0.01%, 0.1% or 1% collagen (groups 2, 3, 4 respectively) or, 10%, 30 % or 50% bioactive glass (groups 5,6,7 respectively), or Mineral Trioxide Aggregate (group 8). The root slices were incubated in tissue culture plates with BHK-21 fibroblast cell line. Phase contrast and scanning electron microscopes were used to score cell quantity, morphology and cell attachment. The data were statistically analyzed by one way ANOVA with Post Hoc Tukey HSD test (p = 0.05).* ***Results and conclusions:*** *Group 5 showed the highest scores which was significantly higher than all other groups (p< 0.05) except group 8, with which there was no significant difference (p> 0.05). Glass ionomer cement with 10% bioactive glass showed better adhesion and spreading of cells than glass ionomer cement with 0.01% collagen. The biocompatibility of collagen and bioactive glass was concentration dependent. The addition of bioactive glass improved the biocompatibility of glass ionomer cement to fibroblasts better than addition of collagen.*

Keywords: *Glass ionomer, collagen, bioactive glass, biocompatibility, fibroblast, BHK-21*
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INTRODUCTION

Root end filling materials are used in surgical endodontics with the objective of obtaining an apical seal, which will prevent movement of microbiota and their by products from the root canal system into the periradicular tissues and vice-versa.[1] An ideal root end filling material should be non-toxic, non- caricogenic and biocompatible with the host tissue. In addition, it should be insoluble in tissue fluids and dimensionally stable. The sealing ability should not be influenced by the presence of moisture and blood. Furthermore it should be easy to manipulate, fast setting and radiopaque.[1] Different materials have been evaluated for this purpose in both *in vitro* and *in vivo* trials, but no material appears to satisfy all these characteristics.[2]

Among the materials studied thus far, mineral trioxide aggregate (MTA) has shown to be superior to other root end filling materials, with favorable tissue response, although it does have its own inherent disadvantages like prolonged setting time, high cost and poor handling properties. Despite the technological advancements in endodontics, location of the surgical site and physical characteristics of MTA has its limitations in placement of this material.[3]

Another material which has been studied for numerous applications in restorative dentistry, is Glass ionomer cement (GIC) which has demonstrated good sealing ability and tissue response.[2] Glass ionomers have been used for perforation repair and retrograde filling where biointegration with periodontal tissue is required. Biocompatibility of glass ionomers has been extensively investigated and conventional glass ionomer cements have shown a more favorable response to gingival fibroblasts and epithelial cells when compared to resin modified counterparts.[4,5] However, reports on the biological compatibility of GIC is not conclusive in that some reports claim that the material is cytotoxic to fibroblasts and macrophages, while some claim the contrary.[6,7]

Recent studies have reported the use of several additives to GIC to improve the biocompatibility of the cement - collagen,[8] hydroxyapatite[9] and bioactive glass[10] in various dental applications including root end filling. Bioactive glass

* Chandana Subbarao, MDS, Senior Lecturer, Department of Conservative Dentistry and Endodontics, Saveetha Dental College and Hospitals, Saveetha University, Chennai, India
** Prasanna Neelakantan MDS, Senior Lecturer, Department of Conservative Dentistry and Endodontics, Saveetha Dental College and Hospitals, Saveetha University, Chennai, India
*** CV Subbarao, MDS, Professor Emeritus, Department of Conservative Dentistry and Endodontics, Saveetha Dental College and Hospitals, Saveetha University, Chennai, India

Send all correspondence to: Dr Prasanna Neelakantan, Plot 1500, 16th Main Road, Anna Nagar West, Chennai, Tamil Nadu, India

Phone : +91-44-98847 54914
Fax: +91 – 044 – 2616 3639

E-Mail: prasanna_neelakantan@yahoo.com

imparts surface activity to the material and helps in forming a bond with mineralized hard tissues such as bone and dentin. It provides a biological seal in form of mineral deposition at the apex. A recent report concluded that 0.01% of collagen added to GIC enhanced its biocompatibility and strength.[9] However, an extensive review of literature showed that there was no comparative study on the biological properties of these modified GIC. It was the aim of this study to evaluate the biological property of GIC impregnated with different concentrations of collagen or bioactive glass, on baby hamster kidney (BHK-21) fibroblasts *in vitro*, in terms of two parameters - (i) cytotoxicity to fibroblasts and (ii) cellular adhesion to tooth structure. MTA was used as the standard for comparison. The null hypothesis was that incorporation of collagen or bioactive glass does not improve the biocompatibility of GIC.

MATERIALS AND METHOD

Human single-rooted maxillary central incisors (n=80) were collected and thoroughly cleaned by removing the hard deposits using curettes and the soft deposits by soaking in 5.25% NaOCl for 10 minutes. The teeth were decoronated at the cemento-enamel junction using a diamond disc, under water-cooling. The root lengths were standardized to 15 mm. The study protocol approved by the Institutional Review Board and Ethical Committee of the University. Apical patency was checked for all the teeth using a size 10 K-file (Mani Inc, Tochigi, Japan). Cleaning and shaping was done using rotary Nickel Titanium instruments (Mtwo, VDW Dental, GMBH) up to an apical size of 40. Obturation was done by lateral compaction technique using 0.02 taper, 40 size gutta-percha as master-cone and 0.02 taper accessory cones (DentsplyMaillefer, Baillagues, Switzerland) using an epoxy resin sealer (AH Plus, Dentsply Maillefer, Baillagues, Switzerland).

The roots were stored in 100% humidity for 24 hours to ensure complete setting of the sealer. Following this, the apical 3 mm of each root was resected perpendicular to the long axis of the root using a sterilized straight fissure bur (No. 245) in low speed hand piece. Root end cavities (3mm) in depth were prepared on the apical side of the roots using inverted cone bur in a high speed hand piece with a water coolant. The cavity depth was determined and standardized by marking the pre-adjusted length of the shank with an indelible marker. The roots were sectioned with a diamond disc 4 mm coronal and parallel to the apically prepared surface to obtain 4mm thick root slices. Ten glass slides (1cm x 1cm) were used as control (5-positive and 5-negative controls).

The root slices were randomly divided into 8 groups (n=10) and were restored with one of the following materials, after sterilizing the specimens in an autoclave: Group 1 – Conventional Glass Ionomer Cement (Fuji II, GC, Tokyo, Japan); Group 2 – Glass Ionomer Cement with 0.01% collagen (Sigma Aldrich, St.Louis, MO, USA); Group 3 – Glass Ionomer Cement with 0.1% collagen; Group 4 – Glass Ionomer Cement with 1% collagen; Group 5 – Glass Ionomer Cement (90%) with 10% bioactive glass (45S5 Nanosized bioactive glass, Swiss Federal Institute of Technology, Zurich, Switzerland); Group 6 – Glass Ionomer Cement (70%) with 30% bioactive glass; Group 7 – Glass Ionomer Cement (50%) with 50% bioactive glass; Group 8 – Mineral Trioxide Aggregate (ProRoot MTA, Dentsply) was used as the standard for comparison.

Bioactive glass was mixed homogeneously with the powder component of glass ionomer by ball milling with zirconia balls while premixed amounts of type I collagen (w/w) (0.01%, 0.1%, 1%) was mixed with the liquid component of glass ionomer under vigorous stirring followed by 20 mins sonication on ice and degassing and kept at 4ºC before use. The specimens were incubated for 72 hours at 37ºC in a humidified atmosphere containing 5% CO_2 and 95% air in an incubator.

Culture of Fibroblast Cell Line

A fibroblast cell line BHK - 21 (Baby Hamster Kidney fibroblasts, ATCC -CCL10) was used in this study. The culture media (GIBCO, G-MEM BHK-21, Invitrogen, CA, USA) and TVG (trypsin, veresine, glucose) were thawed in a water bath at 37ºC. The medium in BHK-21 culture flask was discarded and the flask was rinsed with 1ml of TVG solution. The solution was discarded and 1 ml of fresh TVG solution was added.

The flask was incubated till cells get detached from the flask surface in 5 to 10 min. Then medium trypsinized cells (0.5mL) were added and flushed gently. Another 10mL of medium was added to the flask to get 15mL volume of suspended BHK-21 cells which will have a concentration of 10^3 – 10^5 cells/mL of medium. The flasks were incubated at 37ºC in an incubator. The flasks were observed daily under a inverted phase contrast microscope for monolayer. Once the monolayer was formed, the medium was discarded and minimal essential medium was added to maintain cells in the log phase. The medium was changed at regular intervals to maintain the pH of the medium.

Experimental procedure

The growth of BHK-21 cells was examined in an inverted phase contrast microscope before beginning the experiment (Figure 1). The experiment was performed in a 24-well tissue culture plate. Root slices were placed with the coronal surface contacting the surface of the well. Then 1 mL cell suspension was carefully added over the root slice (experimental groups) or in the well with glass slides (negative control).

For positive control, 0.5 mL of methyl methacrylate (2% vol/vol) was added to the cell suspension before being dispensed into the well to bring about absolute inhibition of cell attachment to the surface of glass slides.[11] The tissue culture plates were incubated at 37ºC in a humidified environment consisting of 5% CO_2 and 95% air in an incubator for 72 hrs. No attempts were made to extend the incubation period beyond 72 hrs as cellular exposure to exogenous materials like contaminated teeth may compromise cellular integrity

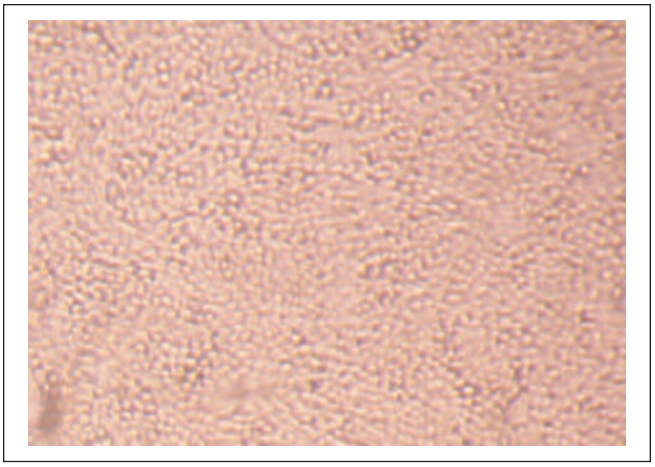

Figure 1. Growth of BHK – 21 cells before beginning the experiment

and lead to inaccurate and misleading results.

On completion of incubation, specimens in all culture plates were observed under a phase contrast microscope and a semi-quantitative analysis was performed using a scoring method of cell quantity and morphology as suggested by Camilleri et al.[12] Score 0: No cells; 1: Occasional round cells; 2: Spare flattened-out cells; 3: Substantial cell growth; 4: Confluence of cells. The quality of cell attachment was assessed using scanning electron microscope. The scoring was done for each specimen by two pre-calibrated observers, who were competent in cytotoxicity assays. The observers were blinded with regards to the materials and groups evaluated. Inter-rater agreement was measured between the two observers by comparison of scores. Intra-rater agreement was measured by having both the observers evaluate one half of the images at each of two separate sessions. A kappa correlation analysis was done to evaluate intra-rater and inter-rater agreement. The data was statistically analyzed by one way analysis of variance with Post Hoc Tukey HSD test. The alpha type error was set at p = 0.05.

SEM Evaluation

On completion of incubation, the specimens were prepared with a few drops of 0.1% of glutaraldehyde in a cocodylate buffer (pH 7.2) for five minutes and then in 2% glutaraldehyde for a period of 30 minutes. The specimens were dehydrated and washed sequentially in a series of 50%, 70%, 90% and 95% ethanol and twice in absolute ethanol for 30 min—before they were critical point dried with carbon dioxide. Specimens were mounted on brass stubs and sputter coated with gold after placing them on copper grid for 3–5 minutes, following which they were viewed under a Scanning Electron Microscope (JSM-6390A Analytical SEM, JEOL, Tokyo, Japan) with an accelerating of voltage of 25KV, operated in the secondary electron mode at 1500x and 3000x magnifications.

RESULTS

There was 100% intra-rater agreement and a test to evaluate examiner reliability was deemed unnecessary. Kappa correlation analysis showed an inter-rater agreement of 0.822. The scores given for the specimens based on the observations of phase contrast images are shown in Table 1. Comparison of the mean values of scores showed that groups 5 and 8 showed significantly higher scores than all other groups ($p<0.05$). This was followed by followed by groups 2 and 6. There was no significant difference between groups 5 and 8 ($p>0.05$). Other groups demonstrated no significant differences ($p>0.05$).

The specimens in group 5 (Figure 2a) and 8 showed complete confluence of cells, while group 2 (Figure 2b) and

Table 1. Scores [Mean ± S.D] of cell quantity and morphology

GROUP	SCORES (MEAN ± S.D)
1 (GIC)	2.00 ± 0.47
2 (GIC + 0.01% COLLAGEN)	3.40 ± 0.51
3 (GIC + 0.1% COLLAGEN)	2.50 ± 0.70
4 (GIC + 1% COLLAGEN)	2.30 ± 0.82
5 (GIC + 10% BAG)	3.70 ± 0.48
6 (GIC + 30% BAG)	3.10 ± 0.87
7 (GIC + 50% BAG)	1.40 ± 0.69
8 (MTA)	3.95 ± 0.62

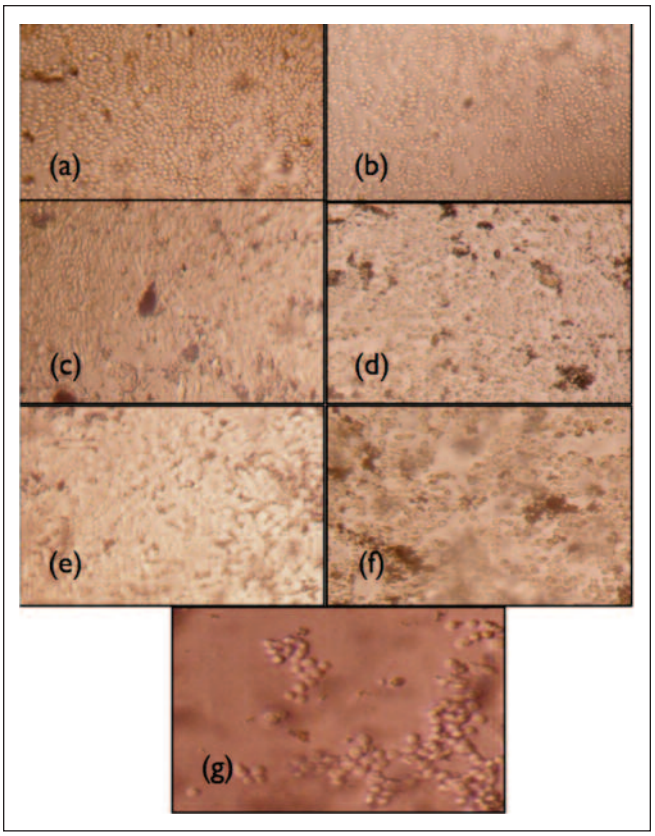

Figure 2. Phase contrast images of fibroblast cells in different groups: [a] Complete confluence of cells in Group 5; [b], [c] Specimens of group 2 and 6 respectively, showing substantial cell growth, mostly flat cells with occasional round cells; [d], [e] Sparsely flattened-out cells in specimens of groups 3 and 4 respectively; [f], [g] Occasional round cells in specimens of groups 7 and 1 respectively.

group 6 (Figure 2c) exhibited substantial growth of mostly flat cells with occasional round cells. Group 3 (Fig. 2d) and group 4 (Figure 2e) exhibited sparsely flattened-out cells, while group 7 (Figure 2f) and Group 1 (Figure 2g) showed occasional round cells. The SEM micrographs of BHK-21 incubated with the different groups are represented in Figure 3 a-n.

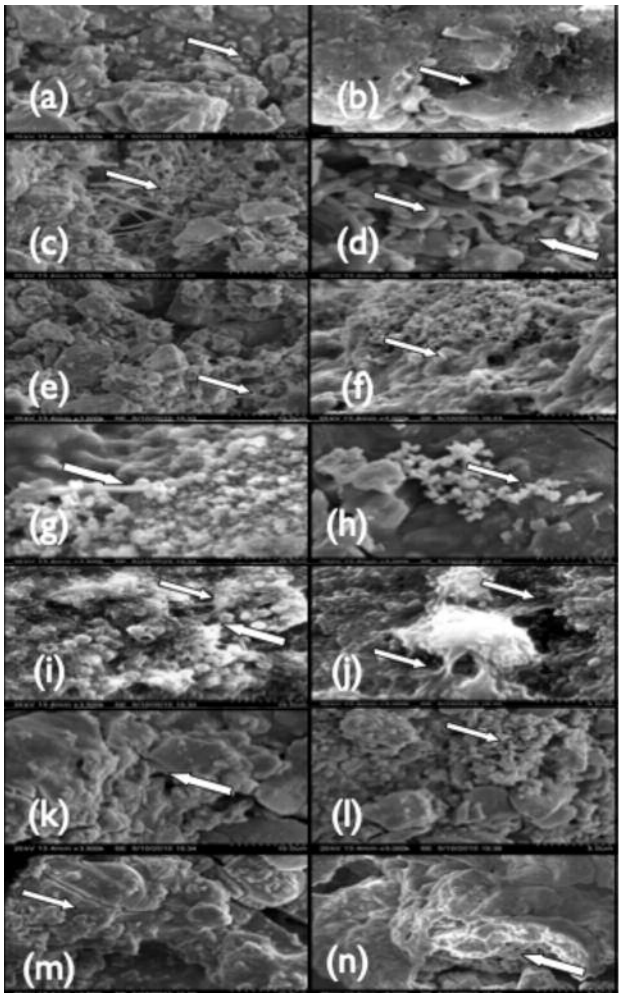

Figure 3. Fibroblast cells in different treatment groups. Value within parenthesis indicates magnification under SEM. [a] Group 1 specimens showing showed round cells with varying degree of degeneration [1.5x]; [b] Presence of blebs and vacuoles on the surface of cells in Group 1 [3x]; [c] Confluence of cells, suggestive of complete proliferation, in specimens of group 2 [1.5x]; [d] Flattened out cells with filopodia and microvilli in group 2 [3x]; [e]Specimen of Group 3 demonstrating few few microvilli [1.5x]; [f] Round cells occupied majority of the cells in group 3, with blebs and vacuoles [3x]; [g] Presence of numerous blebs and vacuoles on the surface of cells in group 4 [1.5x]; [h] Demonstration of blebs and vacuoles in higher magnification, in specimens of group 4 [3x]; [i] Group 5 specimens demonstrating roughly spindle shape cells with smooth surface [1.5x]; [j] Cells in group 5 showing attachment with lamelipodia indicative of complete attachment [3x]; [k] Group 6 showed numerous flattened out cells [1.5x]; [l] Presence of microvilli and filipodia in cells of group 6 [3x]; [m] Cells in group 7 showing round cells with varying degrees of degeneration [1.5x]; [n] Presence of had blebs and vacuoles on the surface of cells of group 7 [3x].

DISCUSSION

The present study compared the biocompatibility of collagen impregnated or bioactive glass impregnated GIC, to fibroblasts considering cell quantity, morphology and cell attachment as criteria. The results showed that MTA (group 8) and GIC impregnated with 10% bioactive glass (Group 5) offered the best substrate for attachment and growth of BHK-21 fibroblasts.

Healing after periradicular surgery necessitates regeneration of the apical attachment apparatus, as well as osseous repair of medullary and cortical bone. It is postulated that undifferentiated mesenchymal cells, fibroblasts and fibroblast-like cells arise from the periodontal ligament and bone surrounding the root end to initiate the healing process.[13] Hence, an ideal root end filling material apart from being just biologically acceptable should also support osteogenesis and cementogenesis. As mentioned earlier, although considered the present gold standard in root end filling materials, MTA offers technical difficulties in placement in surgical sites, apart from the fact that it is expensive.

To enhance the biocompatibility and bioactivity of glass ionomers, several additives have been evaluated. A recent study concluded that incorporation of collagen in GIC demonstrated a concentration dependent effect on the biocompatibility of the material.[8] Type 1 collagen is the most abundant extracellular matrix component which constitutes 90% of organic mass of bone and major collagen in periodontal tissues. The chemical properties of collagen facilitate good solubility in an acidic environment, as it is highly proline rich and basic. Furthermore, this facilitates stable integration of protein with the liquid component of glass ionomers.[14] Therefore, it is possible that collagen as the matrix component of a material may enhance its mechanical properties and also promote tissue interface compatibilities.[8]

The bioactive glass (BAG) 45S5 used in this study consists of amorphous nanoparticles, 20 to 60 nm in size. The leaching of sodium ions and dissolution of calcium, phosphate and silica from the glass gives rise to a Si-rich layer on the material and this layer acts as a template for a calcium phosphate precipitation which binds to bone.[15] It has been suggested that growth of cells on bioactive glass 45S5 indicated enhanced function and differentiation of osteoblastic cells.[16]

In the present study, SEM micrographs of group 8 (MTA) and group 5 (GIC with 10% BAG) revealed roughly spindle shaped cells with smooth surface and lamelipodia attachment indicating complete attachment. There was no significant difference between the negative control group, group 5 and 8 in that, both groups showed fibroblasts with similar morphology and good attachment to the substratum. Specimens of Group 2 (GIC with 0.01% Collagen) showed confluence of cells on the surface suggestive of complete proliferation of cells in which flattened out cells with filopodia and microvilli were observed. This suggests good integration of collagen to glass ionomer, which yielded better results in promoting growth of fibroblasts. These findings

are in agreement with those of Chang et al.[8] SEM micrographs of BHK-21 fibroblasts incubated with root slices containing group 3, 4 and 6 showed few flattened cells with microvilli indicating that the cells had started attaching in their initial stages. Occasional round cells with blebs on the surface were present indicating mild toxic nature of material. Micrographs of specimens of groups 1 and 7 showed round cells with varying degrees of degeneration, evidenced by the presence of blebs and vacuoles on the surface of the cells. Persistence of round cells with little or no spreading could be attributed to the toxicity of the material. These observations clearly indicate the toxic nature of the materials which could be caused by the leachable and toxic components.

The present study demonstrated that Group 1 (GIC) exhibited higher degree of toxicity in comparison with collagen impregnated glass ionomer and bioactive glass impregnated glass ionomer. Hence the null hypothesis must be partially rejected. However, it is important to note that the responses of both the collagen and bioactive glass incorporations were concentration dependent.

Apart from the low pH of the cement during setting and maturation, release of aluminum and fluoride ions from GIC may have stimulatory or inhibitory effect on cells.[17] Although there appears to be no consensus on the biocompatibility of the specific ions, it is generally considered that metal ions are cytotoxic to fibroblasts. An earlier study demonstrated that non-fluoride glasses were least toxic to cells *in vitro*.[18] It is possible that one of the mechanisms by which these hybrids show enhanced biocompatibility is by reducing the fluoride and aluminum ion release. However, the exact mechanism for this hypothesis is yet to be clearly elucidated. This hypothesis was also supported by the finding that GIC with 10% BAG showed better biocompatibility than at higher concentrations. Previous studies have shown that addition of 30% BAG to GIC increases its fluoride release.[19] We speculate that addition of 30% and 50% BAG to GIC increases fluoride release further, increasing its cytotoxic effects. However, further studies are required to determine the bioactivity of these materials.

Among the glass ionomer collagen hybrid groups studied, inhibition of cell growth and reduced cell attachment was observed with higher concentrations of collagen than at lower concentrations. At high concentrations of collagen, spreading and attachment of cells are limited by the availability of free integrins on the cell surface since many of these receptors are bound to the surface ligands. At this point, further spreading of cells is impossible since free integrins receptors are needed at the periphery to allow the cell to expand and bind to the additional areas of the substrate.[20] Glass ionomer cement with 10% bioactive glass showed better adhesion and spreading of cells than glass ionomer cement with 0.01% collagen. It is possible that surface texture and composition of root end filling material played an important role in this regard.

CONCLUSIONS

GIC with 10% bioactive glass and MTA offered the best compatibility to BHK-21 fibroblasts by allowing better adhesion and spreading of cells. Although it may not be possible to directly extrapolate the results of in-vitro cytotoxicity tests with the clinical scenario, it is evident that a test material which consistently induces strong cytotoxic reaction in cell culture tests will also exert toxicity in living tissues. Further research of the biological and mechanical properties of the material is required prior to its endorsement as a root end filling material.

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