RESEARCH ARTICLE

Evaluation and Comparison of the Effect of MTA, MTA Plus, Chitosan, and Their Conjugates on Cell Viability of Human Periodontal Ligament Fibroblasts: An *In Vitro* Study

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Abstract

Aim and objective: The aim and objective of this *in vitro* study was to gauze the effect of MTA, MTA Plus, chitosan, and their conjugates on the cell viability of human pdl fibroblasts.

Materials and methods: A primary culture of human pdl fibroblasts was obtained. Materials used were MTA, MTA Plus, chitosan, and their conjugates. Methyl-thiazol-tetrazolium (MTT) colorimetric assay and Neutral Red assay were used to evaluate the cell viability of the root end filling materials after 24 hours of setting. Optical density of adherent stained biofilm was read at 570 nm using ELISA auto reader. Cell viability was evaluated as percentage of the negative control group, which represented 100% cell viability. Statistical analyses were done with one-way ANOVA and Tukey's post hoc test.

Results: Test indicates that there was no cell cytotoxicity seen in Group I (MTA) and Group II (MTA Plus), whereas mild cytotoxicity was seen in Group III (chitosan). However, in Group IV (MTA–chitosan conjugate) and Group V (MTA Plus–chitosan conjugate), proliferation of fibroblasts was seen.

Conclusion: Chitosan showed a synergistic effect with MTA and MTA Plus when used as a conjugate, as no cytotoxicity was seen in both the conjugates. In fact, there was proliferation of fibroblasts seen in Group IV (MTA–chitosan conjugate) and Group V (MTA Plus–chitosan conjugate).

Keywords: Cell viability, Chitosan, MTA, MTA plus, MTT assay, Neutral red assay.

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Introduction

An important concern for dentists has always been the cytotoxicity of the root end materials, as these materials are always in intimate contact with periapical living tissues. This toxicity can lead to delayed wound healing and degeneration of the periapical tissues.[1,2] After endodontic periapical surgeries, ideal healing includes regrowth of periodontal ligament (PDL) along the resected root surface and also regeneration of alveolar bone.[3] Thus, the way PDL cells behave and react when comes in intimate contact with these root end materials is important.

The main objective is to attain optimal conditions so as to permit healing by the formation of a new periodontal attachment apparatus, PDL, alveolar bone, and cementum overlying the resected root end surface.[4] In order to achieve this healing, it has been suggested to place a root end filling material that not only prevents ingress of bacteria or their by-products but also allows the formation of a normal periodontium across the root end surface.[5] Hence, an ideal root repair material should maintain a sufficient seal; should have the ability to adhere to dentin; should be dimensionally stable, be insoluble in tissue fluids, be radiopaque, be non-resorbable over time, and be easily manipulated; should have adequate working time, quick setting time, and adequate compressibility, and should be biocompatible with human tissues.[6] Numerous materials have been advocated as root end repair materials such as zinc oxide-eugenol-based cements, amalgam, super ethoxybenzoicacid (EBA), Cavit, composite resins, glass–ionomer cements, intermediate restorative material (IRM), Portland-based cements, Gutta Percha, etc.[7] Mineral trioxide aggregate (MTA) was developed in 1993 at Loma Linda University and was recommended for root end filling because of its excellent biocompatibility, antimicrobial properties, and good physical and chemical properties.[8,9] It is a tricalcium silicate-based material consisting mainly of tricalcium aluminate, tricalcium silicate, tricalcium oxide, bismuth oxide, and other mineral oxides. Various studies conducted to evaluate the cytotoxicity of Pro-Root MTA have proven it to be biocompatible.[10] A study concluded that MTA does not cause apoptosis of pulpal and periradicular cells; in fact, it induces proliferation in the dentin–pulp complex.[11] Poor handling properties and longer setting time of MTA resulted in the development of newer materials.

Recently, MTA Plus (Prevest Denpro, Jammu, India) has been introduced to the market, which is significantly cheaper than Pro-Root MTA and has shorter setting time. This material is claimed to

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have a finer particle size than the currently available MTA products. As MTA-Plus has been recently available in the market, there are not many studies reported on this material and there is no study on its biocompatibility, so performing a cytotoxicity evaluation seemed to be necessary.

Another material used in this study with a high medicinal value is chitosan. It is the second most abundant natural biopolymer and is a derivative of chitin. It is a biodegradable natural biopolymer, which is obtained by N-acetylation of chitin. It is nontoxic and non-immunogenic. It has strong antimicrobial properties, accelerates wound healing, alleviates pain, inhibits bacterial growth, and exhibits numerous health-related beneficial effects.[12,13] In many studies, it has also been reported that chitosan enhanced bone healing and showed improved hemostasis, in various animal models.[14,15] Studies must be performed especially on human cells, to ensure that these new endodontic materials are biocompatible as they are frequently used in close contact with living tissues and their toxic compounds may damage the surrounding tissues, interfere on the healing process, or can cause allergic reactions. Chitosan is a novel material; it has interested many researchers around the world, particularly in relation to its ability to be a delivery vehicle.[16]

Therefore, the purpose of the present study was to use chitosan as vehicle with the root end materials in order to investigate the cytotoxicity on human PDL fibroblasts, using an assay that assessed the metabolic activity of cells after exposure to extracts of the test materials.

Materials and Methods

This study was conducted at the Central Research Lab in Maratha Mandal Institute of Dental Sciences and Research Centre, Belgaum. The protocol of the study was approved by the Institutional Review Board and Ethical Committee of the Institution.

In this study, the materials subjected for cell viability assay against the human periodontal cells were MTA, MTA Plus, and their conjugates with chitosan.

Cell Culture

PDL fibroblasts were obtained from the roots of a healthy unerupted mandibular third molar tooth that was extracted from a patient in the Oral Surgery Department at Maratha Mandal College of Dental Sciences. The tooth was placed in Dulbecco's modified Eagle's medium (DMEM—Gibco/Invitrogen Corporation), immediately after extraction under aseptic conditions for cell cultivation. PDL tissues were gently curetted off from the middle third of the root and placed in DMEM containing gentamycin (50 μg/mL) (Sigma, India), streptomycin (100 μg/mL) (Sigma, India), and amphotericin-B (250 μg/mL) (Sigma, India) to prevent contamination, and this medium was referred as complete medium. Cells were then cultivated in 10% fetal bovine serum (FBS) (Gibco) DMEM at 37°C, in a humidified atmosphere of 5% carbon dioxide and 95% air until fibroblast-like cells had grown to confluency, with medium replacement every other day. Prior to experimental tests, the cells were detached, counted, and seeded at 1.25×10^4 cells/well in 96-well plates, and 100 μL medium was added to each well and incubated for 24 hours in an atmosphere of 5% CO2 at 37°C.

Preparation of Materials

The sample size was 14 in each group with an alpha error of 5 and 80% power of test. The materials were divided into five groups:

- Group I—Pro-Root MTA mixed with distilled water
- Group II—MTA Plus mixed with gel
- Group III—2% chitosan gel
- Group IV—MTA mixed with 2% chitosan gel
- Group V—MTA Plus mixed with 2% chitosan gel

Two grams of chitosan was dissolved in 100 mL 0.2M acetic acid to form 2% chitosan gel. This gel was used as vehicle to make conjugates with MTA and MTA Plus for Group IV and Group V. This MTA Plus is supplied with either water or a gel for mixing. Within the current study, gel was used for mixing of MTA Plus and distilled water was used for the mixing of Pro-Root MTA. The materials were mixed in line with the manufacturer's recommendation and then were dissolved in 1 mL of phosphate-buffered saline (PBS) to get a solution. This solution was placed into 96-well tissue culture plates; 200 μL of complete DMEM was placed over each sample; after that, the plates were incubated at 37°C at 100% relative humidity for 24 hours. The medium was then drawn off and sterile-filtered at 0.22 μm.

MTT Assay

Single-cell suspensions of human PDL fibroblasts were seeded in 96-well flat-bottomed plates. 1.25×10^4 cells per well (determined by hemocytometer counting) in complete DMEM and was incubated in humidified atmosphere of air and 5% CO_2 at 37°C for 24 hours. The medium was then replaced with 200 μL aliquots of test extracts, and also, the cells thus exposed were incubated for 24 hours at 37°C under humidified air and 5% CO_2. After exposure, the ability of cells to cleave the tetrazolium salt to formazan dye determines the viability of cells. The medium was removed with a sterile pipette, and 200 μL of PBS was added to individual wells for 1 minute, then replaced with 100 μL of complete medium, and 10 μL of a 5 mg/mL solution of 3-(4,5-dimethylthiazole-2-μL)-2,5-diphenyl tetrazolium bromide (MTT) solution was added to each well. The cells were incubated for 4 hours at 37°C in a 5% CO_2 atmosphere in the MTT/medium solution. One hundred microliters of a 6.25% 0.1 mol/L NaOH in dimethyl sulfoxide (DMSO) solution was added to each well, and then the plates were incubated overnight to solubilize any formazan crystals formed. Plates were shaken for 60 minutes at room temperature to achieve a uniform color. Optical density (OD) was then measured at 570 nm in an exceedingly multiwell spectrophotometer. Mean absorbance values obtained from the DMSO-solubilized formazan for each extract concentration were calculated and then expressed as a percentage of the mean negative control value (set at 100% viability). The percentage of relative cell viability was calculated using the subsequent formula:

$$\text{Cell viability \%} = \text{As} - \text{Ab}/\text{Ac} - \text{Ab} \times 100$$

As—absorbance of sample; Ab—absorbance of blank; Ac—absorbance of control

Neutral Red Assay

Material extracts were replaced by 0.1 mL α-MEM (minimal essential medium) containing 50 μg Neutral Red (NR)/mL followed by incubation at 37°C, 95% humidity, and 5% CO_2 for 3 hours. Then, the content of the well was removed and the wells were washed with sterile PBS and dried. The working solution of Neutral Red (40 mg of Neutral Red indicator to 10 mL of sterile distilled water) was added to the wells and incubated at 37°C, 95% humidity, and 5% CO_2 for 90 minutes. After 2 hours, the colorimetric product was solubilized in 100 μL of an ethanol solution (50% ethanol and 1% acetic acid) and then washed with PBS. The optical densities of the solutions were measured in a spectrophotometer at 570 nm.

The percentage of relative cell viability was calculated using the subsequent formula:

Cell viability % = Mean OD of wells receiving material/ Mean OD of control wells × 100

Data Analysis

Mean absorbance values obtained from the MTT assay and Neutral Red assay for each extract concentration were calculated and expressed as a percentage of the mean negative control value (set at 100% viability). Differences in mean cell viability values between materials were assessed by ANOVA and Tukey's post hoc tests. Statistical software SPSS 20.0 was used at a significance level of 5%.

Results

MTT Assay

As per one-way ANOVA test, the cell viability values of groups were found to be statistically significant amidst all test groups with *p* value 0.0006 (Table 1).

Further pairwise comparisons was made by Tukey's multiple post hoc procedures; a statistically significant difference was seen when Group I (Pro-Root MTA) was compared with Group II (MTA Plus) and Group IV (Pro-Root MTA–chitosan conjugate), whereas for Group II (MTA Plus), a statistical difference was seen when compared with Group III (chitosan), Group IV (Pro-Root MTA–chitosan conjugate), and Group V (MTA Plus–chitosan conjugate) (Table 2).

Neutral Red Assay

As per one-way ANOVA test, the cell viability values of groups were found to be statistically significant amidst all test groups with *p* value 0.0001 (Table 3).

As per pairwise comparisons made by Tukey's multiple post hoc procedures, the following results were obtained using Neutral Red. A statistically significant difference was seen when Group I (Pro-Root MTA) was compared with Group III (chitosan), Group IV (Pro-Root MTA–chitosan conjugate), and Group V (MTA Plus–chitosan conjugate), whereas for Group II (MTA Plus), a statistical difference was seen when compared with Group III (chitosan) and Group V (MTA Plus–chitosan conjugate) (Table 4).

Table 1: Comparison of the cell viability of the groups of MTT Assay by one-way ANOVA

Sources of variation	*Degrees of freedom*	*Sum of squares*	*Mean sum of squares*	*F value*	*p value*
Between groups	5	0.7894	0.1579	4.9195	0.0006*
Within groups	78	2.5031	0.0321		
Total	83	3.2925			

*$p < 0.05$

Table 2: Pairwise comparison of the cell viability of the groups of MTT Assay by Tukey's multiple post hoc procedures

Groups	*Group I*	*Group II*	*Group III*	*Group IV*	*Group V*	*Control*
Mean	0.6367	0.8321	0.5352	0.5927	0.6144	0.7244
SD	0.2809	0.2352	0.1365	0.0858	0.1232	0.1311
Group I	–					
Group II	$p = 0.0500$*	–				
Group III	$p = 0.6659$	$p = 0.0006$*	–			
Group IV	$p = 0.0004$*	$p = 0.0088$*	$p = 0.9571$	–		
Group V	$p = 0.9995$	$p = 0.0226$*	$p = 0.8502$	$p = 0.9996$	–	

*$p < 0.05$

Table 3: Comparison of the cell viability of the groups of Neutral Red by one-way ANOVA

Sources of variation	*Degrees of freedom*	*Sum of squares*	*Mean sum of squares*	*F value*	*p value*
Between groups	4	0.4573	0.1143	9.5314	0.0001*
Within groups	65	0.7796	0.0120		
Total	69	1.2369			

*p value < 0.005

Table 4: Pairwise comparison of the cell viability of the groups of Neutral Red by Tukey's multiple post hoc procedures

Groups	*Group I*	*Group II*	*Group III*	*Group IV*	*Group V*
Mean	1.0328	1.0520	1.2047	1.0675	1.2229
SD	0.1543	0.0602	0.0174	0.1483	0.1012
Group I	–				
Group II	$p = 0.9905$	–			
Group III	$p = 0.0010$*	$p = 0.0042$*	–		
Group IV	$p = 0.0006$*	$p = 0.9958$	$p = 0.0127$*	–	
Group V	$p = 0.0003$*	$p = 0.0011$*	$p = 0.9922$	$p = 0.0034$*	–

*p value <0.005

Discussion

Varieties of tests are available to evaluate the cytotoxicity of dental materials in cultured mammalian cell populations.[16] Functional assays evaluate the capability of the cell to supply the required energy for anabolic activities, or the end products of such activities. In the present study, tetrazolium salt 3-(4,5-dimethylthiazole-2-µL)-2,5-diphenyl tetrazolium bromide (MTT) was used to measure mitochondrial dehydrogenase activity.[17] It consists of a straw-colored substrate that produces an end product. When cleaved by active mitochondria, the end product consists of deep blue formazan crystals; hence, the reaction occurs only in metabolically active and living cells.

The criteria to select an assay should be based on its compatibility with the chemical nature of the material being tested. For instance, a permeability assay is unlikely to determine the cytotoxicity of a material which does not cause a change in cell membrane permeability. As MTA is a hydrophilic material, it's less likely to release ionic components, whereas it's more likely to interfere with intracellular enzyme activities rather than influencing membrane permeabilities.[16] Therefore, MTT assay was chosen for the present study. However, few dyes are also used to test the cytotoxicity of dental materials where the dead cells take up the dye, such as trypan blue or neutral red, which is more sensitive and can provide the percentage of viable cells. Hence, neutral red assay was used as the second test to determine the cell cytotoxicity.

During the designing of an *in vitro* biocompatibility study, the most important issue is to consider the cell type. Some of the cell types that are usually investigated are human fibroblasts, osteoblasts, and mouse fibroblasts. It's convenient to culture human cells with a lower number of passages leading to minimal cell changes due to cell culture manipulation. Human PDL cells were chosen for this study because it provides additional advantage of reducing bias concerning non-tissue-specific cell lines and species origin.[1,2]

Root end filling materials were chosen based on the fact that these cements release soluble components which when placed into the root end cavities get diluted by tissue fluids and are carried to the surrounding tissues and cells. MTA was developed as a root end filling material, which suggests that it is designed for repair and surgical purposes.[9] A large number of studies describe the properties of MTA; especially, its biocompatibility is compared with other root end filling material.[18–20] MTA was found to be biocompatible when tested with human PDL fibroblasts for apoptosis, cell viability, and mitochondrial dehydrogenase activity.[21,22] Despite this fact, Ma et al. in their *in vitro* study noticed a dose–response effect of MTA on cell toxicity.[23] Another material used in this study is MTA Plus. As there is limited literature regarding biocompatibility of MTA Plus, in this study, the biocompatibility of MTA Plus was evaluated in comparison with MTA. One among MTA's major components, within the presence of water is calcium hydroxide, which increases the surrounding pH.[23] This alkaline pH has a destructive effect on protein structures and may promote cell membrane damage and also enzyme denaturation.[20] This might be the explanation behind MTA's cytotoxicity.

Chitosan was used in the present study as a vehicle. Cell growth reached a plateau at a concentration of 0.1 mg/mL, whereas the inhibition of cell growth was observed above this concentration.[2] Thus, the concentration of chitosan utilized in this study was 0.1 mg/mL. Chitosan has similar structural characteristics to those of the glycosaminoglycans and mucopolysaccharide, and mimics their functional behavior. Mucopolysaccharides carry out the migration and proliferation of progenitor cells, thereby increasing tissue regeneration, while collagen impairs regeneration and reduces cellular migration. Previously conducted studies have stated that chitosan can be used as a substrate that enhances the differentiation and migration of osteoblasts, and conversely, might decrease the function of fibroblasts, hence indirectly facilitating osteogenesis.[24] In an addition to its biological properties, chitosan has excellent structural characteristics due to which it could also be even utilized in other clinical applications such as bone substitute or scaffold for cell attachment. Many studies have also demonstrated that chitosan could be used as an effective scaffold for growth factors.[25,26] However, the pH of chitosan is 4, and when used with MTA and MTA Plus as a conjugate, it has shown synergistic effect, thus reducing the cytotoxicity of the individual materials.

Qualitatively, results of the growth measurement were obtained by examining the cells under phase contrast microscope at 10× magnification. However, the proliferation of fibroblasts was seen in both the conjugates, i.e., Group IV (MTA–chitosan conjugate) and Group V (MTA Plus–chitosan conjugate). There was no cytotoxicity or cell lysis seen in Group I (MTA) and Group II (MTA Plus). However, mild cytotoxicity or cell lysis was seen in Group III (chitosan). In our study, the results from the investigation of these materials reveal that there's no significant statistical difference between the cytotoxicity of MTA (Group I) and MTA Plus (Group II). The reason might be that the mechanism of setting is the same for both the cements. Deus et al., reported similar results where cytotoxicity of Pro-Root MTA, Portland Cement, and MTA Angelus was carried out on human ECV 304 endothelial cell lines.[27] The findings of other research studies too support the results of the present study.[28,29] However, the conjugates in our study showed positive results, i.e., the conjugates showed a lesser cytotoxicity when compared with their counterparts. When analyzed statistically, there was a significant difference seen in the cytotoxicity of Group I (MTA) and Group II (MTA Plus) when compared with Group III (chitosan), i.e., the cell viability percentage of chitosan was better when compared with the materials. Further, there was statistically significant difference seen between Group I (MTA) and Group IV (MTA–chitosan conjugate), and similar results were seen in between Group II

(MTA Plus) and Group V (MTA Plus–chitosan conjugate), i.e., the conjugates of the materials have shown lesser cytotoxicity or better cell viability as compared with their individual counterparts. In fact, there was proliferation of fibroblasts seen in Group IV (MTA–chitosan conjugate) and Group V (MTA Plus–chitosan conjugate). The reason might be the synergistic effect of chitosan with these materials.

Within the limitation of the study, it was concluded that the novel, nontoxic, biodegradable natural polymer—chitosan, proved it's claim, acts in synergism and decreases the cytotoxicity of MTA and MTA Plus when used in conjugation with it. Further studies need to be conducted to evaluate other properties of chitosan alone as well as in conjugation with other novel materials.

Clinical Significance

Chitosan, a novel material, when conjugated with MTA or MTA Plus was relatively less cytotoxic as compared with individual material. Hence, this conjugate will help in faster healing. Also, other studies performed by the author also dictate their good antimicrobial activity and handling properties. Hence, this conjugate can be alternate and better replacement to MTA or MTA Plus.

Conclusion

Within the constraints of this study, it was noted that:

MTA and MTA Plus both are biocompatible root end filling materials which do not show cytotoxicity on PDL fibroblasts.

- Chitosan can be used as a novel material in dentistry, and its various properties can be used to enhance the properties of present materials, as this material was not cytotoxic on PDL fibroblasts.
- Only cell lysis or proliferation was evaluated in the present study, so further studies can be done to evaluate the cell lineages to which these cells are proliferating into.

Thus, the findings opened new opportunities for the use of chitosan alone or in combination to improve the bioactivity of dental materials and beyond.

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