Histological evaluation of pulpal response to direct pulp capping using statins with α-tricalcium phosphate and mineral trioxide aggregate in human teeth

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Abstract

Background: The purpose of this study was to compare the outcome of direct pulp capping by statins with α-tricalcium phosphate (α-TCP) and mineral trioxide aggregate (MTA) on pulp tissue of human teeth through histological evaluation.

Aims: The aim of the present study is to compare the pulpal response of statins (simvastatin or atorvastatin) with α-TCP to that of MTA on human teeth by light microscopic histological evaluation.

Materials and Methods: Ninety intact premolar teeth scheduled for orthodontic extraction were used for the study. Class 1 cavities were prepared, and the pulp was mechanically exposed under rubber dam isolation. The samples were divided into three groups of thirty teeth each (Group I – simvastatin + α-TCP, Group II – atorvastatin + α-TCP, and Group III – MTA), and the test materials were placed accordingly. After the experimental periods of 7, 30, and 90 days, the teeth were extracted atraumatically. The samples were then evaluated for the degree of inflammation, tissue damage, and hard tissue formation under light microscope, and they were scored based on the histopathologic findings.

Statistical Analysis: The results were analyzed statistically using the Chi-square test.

Results: There was no statistically significant difference between Groups I, II, and III in terms of inflammation, tissue damage, and hard tissue formation for all the three observation periods (P > 0.05).

Conclusions: Simvastatin and atorvastatin with α-TCP were found to be effective in inducing dentin bridge formation which was comparable to MTA.

Keywords: Atorvastatin; pulp capping; simvastatin; α-tricalcium phosphate

INTRODUCTION

A number of factors have been shown to have an impact on the success of direct pulp capping (DPC). The ideal pulp-capping agent should be the one that should stimulate reparative dentin formation, completely resorb, and not affect pulpal tissue vitality in the healing process, not unleash any adverse effects when used, and have good sealing ability.[1]

The introduction of mineral trioxide aggregate (MTA) by Torabinejad et al. (Loma Linda University) in 1993 has resulted in more predictable outcome for DPC procedures. MTA has been reported to be biocompatible in studies with cell culture techniques and connective tissue reactions.[2]

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however, its main disadvantages are handling properties, discoloration, and relatively long setting time.

Statins, inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, are widely used to decrease the hepatic biosynthesis of cholesterol. However, additional pleiotropic effects of statins such as increased bone formation, anti-inflammatory effects, and enhanced angiogenesis due to pro-angiogenic effect have been reported. Furthermore, statins have been reported to stimulate the expression of bone anabolic factors such as vascular endothelial growth factor (VEGF) and bone morphogenetic protein-2 and to promote osteoblast differentiation and mineralization in MC3T3-E1 cells. Simvastatin increased alkaline phosphatase (ALP) activity and osteocalcin (OCN) expression levels in human bone marrow stem cells. Atorvastatin has been reported to increase the number of circulating endothelial progenitor cells and inhibit inflammatory cells and matrix metalloproteinases (MMPs), which play a role in the connective tissue destruction in periodontal disease.

A new material similar to hydroxyapatite has been widely studied for the reconstruction of osseous defects in orthopedic surgeries, neurosurgery, and maxillofacial surgeries. It is the alpha-tricalcium phosphate (α-TCP) bone cement that is an apatite carbonate formed by the mixture of the α-TCP with calcium carbonate and monocalcium phosphate monohydrate powder. There are typically two crystalline phases of tricalcium phosphate, the alpha-phase (α-TCP) and the beta-phase (β-TCP).

The α-TCP is more soluble and biodegradable than β-TCP. The high solubility of α-TCP is desirable for a scaffold in drug-releasing systems. It was speculated that α-TCP when used as a carrier for simvastatin slowly and constantly releases simvastatin. The mineralization and messenger RNA expression of markers (i.e., dentin sialophosphoprotein, dentin matrix protein 1, bone morphogenetic protein 2 [BMP-2], ALP, and osteonectin) of α-TCP containing simvastatin and α-TCP containing atorvastatin-treated cells were comparable with MTA-treated cells.

However, there are no in vivo studies to evaluate the effect of statins and α-TCP in dentin regeneration. Therefore, this study aimed to evaluate the effect of various statins with α-TCP as a carrier on the pulp tissue of human teeth when used as a DPC agent. The null hypothesis tested was that there was no difference in the pulpal response to statins and α-TCP with that of MTA in terms of inflammation, tissue damage, and hard tissue formation.

**MATERIALS AND METHODS**

A total of 90 intact, noncarious maxillary and mandibular premolar teeth of young adult patients (age group: 18–24 years; 12 male and 10 female patients with 4 teeth each and 1 male patient with 2 teeth) scheduled for extraction of premolars for orthodontic reasons were selected for the study. The teeth with the following criteria were excluded from the study: decayed or fractured teeth, previous restorative history, symptomatic teeth, teeth with any other pathology, teeth not conducive for rubber dam isolation, negative response to pulp vitality testing, and presence of systemic diseases.

All experimental protocols were reviewed and approved by the Institution Ethics Committee (R. C No. 0430/DE/2015).

The participants signed the informed consent forms after they received a thorough explanation regarding the experimental rationale, clinical procedure, and possible risks. All the patients were recruited by a single operator.

**Preparation of simvastatin and atorvastatin containing α-tricalcium phosphate**

Simvastatin and atorvastatin are dissolved in absolute ethanol and absolute methanol, respectively. The ethanol and methanol are allowed to volatize to get a homogeneous powder form. Then, 0.5 mg of simvastatin or 0.5 mg of atorvastatin was added to 70 mg of α-TCP for each tooth.

**Clinical procedure**

All teeth were examined clinically and radiographically to ensure the absence of caries and periapical pathosis. Preoperative clinical and intraoral assessment was done to evaluate oral hygiene status, presence of caries, tenderness on percussion, periodontal status, and restorability of the tooth.

Radiographic evaluation was done using digital radiography (CS Imaging Software Version 7, Carestream) with XCP film positioning device using paralleling technique and standard exposure modes of 100 ms, 4 mA, and 60 kVp.

Electrical pulp testing was performed to assess the vitality before the experimental procedure. Calculus and debris were removed from tooth surfaces. After administering local anesthesia, 2% lidocaine, and rubber dam isolation, the teeth were cleaned with a rubber cup and prophylactic paste at low speed. This site was then washed with 2% chlorhexidine.

Occlusal cavities of dimensions approximately 1.5 mm wide buccolingually, 2 mm long mesiodistally, and about 2.5 mm deep were prepared using a sterile straight fissure diamond (SF-11, Mani Inc., Japan) at a high speed under water/spray coolant. The cavity walls were washed with normal saline. Then, a pulp exposure was made in the center of the floor of the cavity using a no. 2 round tungsten carbide bur (diameter 0.5 mm) at high speed.
without penetrating the pulp space. New sterilized burs were used for each procedure.

Hemorrhage was controlled with a sterile cotton pellet soaked in saline.

The prepared teeth were randomly divided into three groups (n = 30 each; 10 per observation period) depending on the pulp-capping material used. Randomization was carried out by the sealed-envelope method. Opaque envelopes containing a slip that mentions Group I, Group II, or Group III were numbered according to the randomization schedule. The randomization was done by a third person other than the operator or the investigator.

- In Group I: 0.5 mg simvastatin and α-TCP (Sigma-Aldrich, St. Louis, Missouri, USA) mixed with distilled water
- In Group II: 0.5 mg atorvastatin and α-TCP (Sigma-Aldrich, St. Louis, Missouri, USA) mixed with distilled water
- In Group III: MTA (Angelus, Londrina, PR, Brazil).

After the placement of the respective experimental materials, the cavities were restored using glass-ionomer cement (GC Fuji IX GP). Postoperative intraoral periapical radiographs were taken to confirm the placement of the test material and restorative material. Patients were prescribed ibuprofen 400 mg twice daily for 3 days and were advised to take it if pain arises.

**Specimen extraction**

All the experimental teeth were carefully followed up and clinically assessed before extraction. The extractions were scheduled after 7 days, 30 days, and 90 days. The extraction was carried out atraumatically under local anesthesia of 2% lidocaine in 1:80,000 adrenaline.

**Histological examination**

Immediately after the atraumatic extraction procedure, the roots were sectioned midway between root apex and CEJ and were placed in 10% formalin for 96 h for fixation. After 96 h, the specimens were decalcified in 5% nitric acid, dehydrated in increasing concentrations of aqueous ethanol, and embedded in paraffin wax. Then, serial buccolingual sections of four-micron thickness were cut longitudinally through the center of the exposure site, with a soft-tissue microtome (Leica Microsystems, Germany). The sections were then mounted on glass slides and stained with hematoxylin and eosin. The sections were blindly evaluated by an experienced pathologist under a light microscope (Olympus Light Microscope) at ×40 magnification and calibrated according to the following criteria given by Lu et al. [10] [Table 1].

**Statistical analysis**

The results of the histopathology evaluation were statistically analyzed using the Chi-square test using SPSS software version 16. P < 0.05 was considered statistically significant [Tables 2-4].

**RESULTS**

No apparent adverse events were observed during the experimental periods. None of the teeth were associated with sensitivity or pain during the period of study. All restorations were clinically acceptable without fractures or marginal discoloration at the time of extraction.

In the 7-day samples, Groups I, II and III showed mild or no inflammatory response, normal tissues, or mild odontoblastic disorganization [Figure 1a-c]. At 1 month, most of the samples showed no or mild inflammatory responses and about 50%–70% of the samples showed mild odontoblastic disorganization and either a thin dentin bridge or hard tissue deposition on the lateral walls of the cavity [Figure 1d-f]. At 3 months, majority of the samples in all three groups showed no inflammation, approximately 10%–20% of samples showed mild odontoblastic disorganization, and all the samples showed scores of 3 and 4 for formation of hard tissue [Figure g-i].

The results showed that there is no statistically significant difference between the groups in terms of inflammation, tissue damage, and hard tissue formation as the P value calculated for each of the groups was above 0.05.

**DISCUSSION**

Dentin pulp engineering techniques using materials having progenitor cell potentials determine the success of vital
pulp therapy. The repair response of the tooth is tertiary dentin deposition which could be either reactionary or reparative in nature.

MTA is comprised of calcium oxide in the form of tricalcium silicate, dicalcium silicate, tricalcium aluminate, and bismuth oxide for radiopacity. Unlike CaOH, MTA has a significant advantage in that it provides an excellent seal to tooth structure. Few other human studies found that MTA treatment was more effective for pulp capping. However, its main disadvantages are its handling properties, a relatively long setting time, high cost, and release of potentially hazardous bismuth and aluminum. To overcome these, there exists a continuous search to identify a reliable nonabsorbable bioactive pulp-capping material that consistently stimulates cellular repair mechanisms, seals the dentin, and promotes the formation of a biologically stable reparative dentin bridge.

Simvastatin is a derivative of lovastatin, with a molecular formula: C<sub>25</sub>H<sub>38</sub>O<sub>5</sub> derived synthetically from a fermentation.

| Table 2: Chi-square test for inflammation for all the three intervals |
|--------------------------------------------------|
| Interval | Histopathological scores | Group I Simvastatin + α-TCP | Group II Atorvastatin + α-TCP | Group III MTA | χ² | P |
|----------|---------------------------|-----------------------------|-------------------------------|----------------|-----|-----|
| 7 days   |                           |                             |                               |                |     |     |
| 1        | 1                         | 5                           | 6                             | 6              | 0.271 | 0.873 |
|          | 2                         | 5                           | 4                             | 4              |     |     |
|          | 3                         | 0                           | 0                             | 0              |     |     |
|          | 4                         | 0                           | 0                             | 0              |     |     |
| 1 month  |                           |                             |                               |                |     |     |
| 1        | 1                         | 7                           | 6                             | 6              | 0.287 | 0.866 |
|          | 2                         | 3                           | 4                             | 4              |     |     |
|          | 3                         | 0                           | 0                             | 0              |     |     |
|          | 4                         | 0                           | 0                             | 0              |     |     |
| 3 months |                           |                             |                               |                |     |     |
| 1        | 1                         | 8                           | 9                             | 10             |     |     |
|          | 2                         | 2                           | 1                             | 0              |     |     |
|          | 3                         | 0                           | 0                             | 0              |     |     |
|          | 4                         | 0                           | 0                             | 0              |     |     |

TCP: Tricalcium phosphate, MTA: Mineral trioxide aggregate

| Table 3: Chi-square test for tissue damage for all the three intervals |
|--------------------------------------------------|
| Interval | Histopathological scores | Group I Simvastatin + α-TCP | Group II Atorvastatin + α-TCP | Group III MTA | χ² | P |
|----------|---------------------------|-----------------------------|-------------------------------|----------------|-----|-----|
| 7 days   |                           |                             |                               |                |     |     |
| 1        | 1                         | 5                           | 6                             | 7              | 0.833 | 0.659 |
|          | 2                         | 5                           | 4                             | 3              |     |     |
|          | 3                         | 0                           | 0                             | 0              |     |     |
|          | 4                         | 0                           | 0                             | 0              |     |     |
| 1 month  |                           |                             |                               |                |     |     |
| 1        | 1                         | 5                           | 4                             | 3              | 0.833 | 0.659 |
|          | 2                         | 5                           | 6                             | 7              |     |     |
|          | 3                         | 0                           | 0                             | 0              |     |     |
|          | 4                         | 0                           | 0                             | 0              |     |     |
| 3 months |                           |                             |                               |                |     |     |
| 1        | 1                         | 8                           | 9                             | 8              |     |     |
|          | 2                         | 2                           | 1                             | 2              |     |     |
|          | 3                         | 0                           | 0                             | 0              |     |     |
|          | 4                         | 0                           | 0                             | 0              |     |     |

TCP: Tricalcium phosphate, MTA: Mineral trioxide aggregate

| Table 4: Chi-square test for hard tissue formation for all the three intervals |
|--------------------------------------------------|
| Interval | Histopathological scores | Group I Simvastatin + α-TCP | Group II Atorvastatin + α-TCP | Group III MTA | χ² | P |
|----------|---------------------------|-----------------------------|-------------------------------|----------------|-----|-----|
| 7 days   |                           |                             |                               |                |     |     |
| 1        | 1                         | 10                          | 10                            | 10             |     |     |
|          | 2                         | 0                           | 0                             | 0              |     |     |
|          | 3                         | 0                           | 0                             | 0              |     |     |
|          | 4                         | 0                           | 0                             | 0              |     |     |
| 1 month  |                           |                             |                               |                |     |     |
| 1        | 1                         | 0                           | 0                             | 0              | 0.833 | 0.659 |
|          | 2                         | 4                           | 5                             | 3              |     |     |
|          | 3                         | 6                           | 5                             | 7              |     |     |
|          | 4                         | 0                           | 0                             | 0              |     |     |
| 3 months |                           |                             |                               |                |     |     |
| 1        | 1                         | 0                           | 0                             | 0              | 0.480 | 0.787 |
|          | 2                         | 0                           | 0                             | 0              |     |     |
|          | 3                         | 3                           | 3                             | 2              |     |     |
|          | 4                         | 7                           | 7                             | 8              |     |     |

TCP: Tricalcium phosphate, MTA: Mineral trioxide aggregate.
product of the fungus *Aspergillus terreus*. Hydrolyzed in vivo to an active metabolite, simvastatin competitively inhibits hepatic HMG-CoA reductase, the enzyme which catalyzes the conversion of HMG-CoA to mevalonate, a key step in cholesterol synthesis. Atorvastatin is a pyrrole and heptanoic acid derivative with the empirical formula C\textsubscript{33}H\textsubscript{35}FN\textsubscript{2}O\textsubscript{5}.

Suppression of the enzyme HMG-CoA reductase and the resultant obstruction of the mevalonate pathway is probably the most important mechanism of prevention of bone resorption by statin. Apart from cholesterol, there are a number of other products of this pathway. Apart from cholesterol, there are a number of other products of this pathway. These include compounds referred to as isoprenoids\textsuperscript{[17]}, which are primarily responsible for the prenylation of GTP-binding proteins, and involved in cytoskeletal function and vesicular trafficking.

Thus, bone-resorbing activity is affected because of the interference in the generation of isoprenoids, which causes a disruption of vesicular fusion and ruffled border formation of osteoclasts. The effect of blockage of the mevalonate pathway is explained by the fact that the action of statins on bone is inhibited or reversed by products of this pathway\textsuperscript{[18]}. In addition to inducing bone formation,\textsuperscript{[16,19,20]} it also exhibits certain pleiotropic effects such as anti-inflammatory, induction of angiogenesis, and neuroprotective function.

The successful use of statins to promote bone formation in vivo depends on the local concentration, and there have been continuous efforts to find an appropriate delivery system. An appropriate carrier should be biocompatible, nontoxic, biodegradable, and bioactive, should localize and retain the molecule to the site of application, should serve as a matrix and substrate for cell filtration, cell growth, and differentiation, and should have biomechanical features such as optimal tensile, compressive, and flexural strength.

Tricalcium phosphate is a biodegradable ceramic. Kihara et al.\textsuperscript{[21]} have reported that α-TCP works as an osteoconductive and biodegradable scaffold for bone regeneration maintaining the regeneration space in rabbit parietal bone defects. Yamada et al.\textsuperscript{[22]} have further demonstrated that α-TCP works as a more favorable degradable scaffold compared to β-TCP on rabbit parietal bone. This material has a porous structure.

Simvastatin and atorvastatin are hydrophobic. Simvastatin exhibits its complete solubility in ethanol whereas Atorvastatin is only partially soluble in ethanol. Atorvastatin exhibits complete solubility in methanol. Therefore, stock

**Figure 1:** H and E staining evaluation of effects of simvastatin + α-TCP, atorvastatin +α-TCP, mineral trioxide aggregate on direct pulp-capping assay (×10). (a-c) Areas of inflammation (arrow-inflammatory cells). (d-f) dentin bridge formation after 1 month of material placement; arrow: Dentin deposition on lateral walls. (g-i) complete dentin bridge formation 3 months after material placement (as shown by arrow).
solutions were prepared by dissolving simvastatin and atorvastatin in ethanol and methanol, respectively. Nyan et al. suggested that 0.1 mg of simvastatin mixed with 14 mg of α-TCP was effective for release of simvastatin. They reported that this combination was found to have initial rapid release in 24 h, followed by slow and constant release of simvastatin for 14 days. It is likely that the releasing mechanism at constant rate is due to mechanically trapping the hydrophobic compound in the pores of the material.

Varalakshmi et al. have shown that the combination of 0.5 mg simvastatin with 70 mg α-TCP promoted odontogenic differentiation of human dental pulp stem cells in vitro. Therefore, 0.5 mg of simvastatin was dissolved in ethanol and mixed with 70 mg of α-TCP. Similarly, 0.5 mg of atorvastatin was dissolved in methanol and mixed with 70 mg of α-TCP in this study. Here, α-TCP was used as the carrier for simvastatin and atorvastatin.

In this study, the experimental materials were tested with MTA as it has been found to be promising material due to its biocompatibility and induction of odontogenesis.

Preventing microorganisms from entering the pulp and control of bleeding are key factors for successful DPC. Hence, in the present study, strict isolation with rubber dam application was done to prevent microbial leakage, and complete hemostasis was achieved by applying pressure with cotton pellet moistened with sterile saline.

Direct composite restorations provide the best seal of restorations against coronal leakage after DPC. Although immediate etching and bonding of MTA can be done, Glass Ionomer cement (GC Fuji IX GP) has been used as final restoration after placement of test material in this study because the effects of etching and bonding on the Statins and α+TCP test material is still not known. Glass-ionomer cement with a faster setting time of 6 min when used as an immediate definitive restoration after pulp capping is shown to have better prognostic value as it provides a bacterial tight seal.

To appreciate the success of the pulp-capping procedure, it should be well supported both clinically and histologically. Although a symptom-free tooth with normal function, sensitivity, and radiographic appearance suggests a clinical triumph, only the histological evidence can shed light on the inflammation during the course of healing.

In 7-day observation period, most of the samples in Group I (simvastatin + α-TCP), Group II (atorvastatin + α-TCP), and Group III (MTA) showed mild inflammatory response with very few inflammatory cells infiltrating the exposed area. A mild inflammatory response is essential as a part of normal pulp-healing response. This is most commonly due to operative trauma. There was no significant difference between the groups in terms of inflammation after 7 days.

Very mild inflammation in 7-day observation seen in Group I and Group II can be due to the anti-inflammatory and immunomodulatory actions of statins. They are known to inhibit intercellular adhesion molecule 1 and monocyte chemotactic protein 1 (MCP-1). These molecules are known to attract leukocytes and monocytes to the site of inflammation mediated by adhesion and transmigration. Further statins are also known to decrease T-cell proliferation, reduce the expression of major histocompatibility complex II on antigen-presenting cells, and reduce inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 and IL-8. Simvastatin has a suppressing effect on lipopolysaccharide-stimulated inflammatory cytokines, cell adhesion molecules, and nuclear factor-kB transcription factors on HDPCs.

In 30-day observation period, a reduction in the inflammatory response and re-organization of the pulp tissue was evident in Groups I, II, and III. All the samples during the 7-day observation period showed a zone of tissue destruction which consisted of dispersed dentin particles along with the test material and a zone of pulp tissue damage. This tissue destruction was mainly due to mechanical trauma during the pulp-capping procedure. In 30-day observation period, formation of a thin or partial dentin bridge was evident in all the samples in Groups I, II, and III. This represents a positive healing outcome.

Pulp tissue contains a large amount of blood vessels and peripheral nerves. Statins are known to induce angiogenesis and to regulate the survival and increase neurogenesis of neuronal cells, indicating the possible effectiveness of statins in pulp regeneration along with dentin regeneration. Various mechanisms have been proposed by which statins are known to induce bone and dentin formation.

Statin drugs are known to enhance the expression of VEGF, a bone anabolic factor, in osteoblasts. Simvastatin regulates osteoblast function by increasing the expression of bone sialoprotein, OCN, and type I collagen, as well as suppressing the gene expression of collagenases such as MMP-1 and MMP-13. Simvastatin supports BMP-2-induced osteoblast differentiation through antagonizing TNF-α to Ras/Rho/MAPK pathway and augmenting BMP-Smad signaling.

It promotes differentiation of odontoblasts by expression of angiogenic factors via heme oxygenase-1 pathway. Simvastatin induces in vitro osteogenesis by induction of estrogen-receptor-α (ER-α). It causes upregulation of ER-α, which is normally expressed during osteogenesis leading to
increased recruitment of osteoblasts and subsequent bone deposition.\textsuperscript{31} Simvastatin also prevents isoprenylation which affects the activity of small G-proteins of Ras super family and their subsequent function.\textsuperscript{32} Simvastatin increases the mRNA expression of BMP-2 in osteoblasts, with a subsequent increase in bone formation.\textsuperscript{33} Simvastatin significantly increases ALP activity and mineral nodule formation.\textsuperscript{34} It was observed that simvastatin produced more significant changes in the hemostatic parameters as compared to atorvastatin, while in lipid parameters, atorvastatin was more effective than simvastatin. There was no significant difference between Groups I, II, and III in terms of inflammation, tissue damage, and hard tissue formation in all the three experimental periods in the present study.

A shortcoming of this study is the use of sound teeth. In a germ-free environment, the pulp demonstrated the ability to heal and deposit additional dentin material. In the presence of bacteria, pulpal demise was inevitable. There is no doubt that the results of this study do not reveal the true effects of used biomaterials in more close to real circumstances when the pulp is already inflamed. It is highly recommended to conduct more studies and randomized clinical trials using statins as a pulp-capping agent on carious exposed teeth and longer follow-up duration to substantiate the same.

**CONCLUSIONS**

Within the limitations of this study, it can be concluded that the pulp-healing potential of simvastatin with $\alpha$-TCP and atorvastatin with $\alpha$-TCP in terms of inflammation, tissue damage, and hard tissue formation was comparable to that of MTA at all the experimental periods. Hence, this combination of statins with $\alpha$-TCP can be used as a promising alternative as a pulp-capping agent.

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**Conflicts of interest**

There are no conflicts of interest.

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