Histologic comparison of direct pulp capping of rat molars with MTA and different concentrations of simvastatin gel

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Abstract: Previous in vitro studies have suggested that simvastatin can be used as a direct pulp capping material due to its ability to induce odontoblastic differentiation and angiogenesis. The aim of this animal study was to evaluate the pulpal response to mineral trioxide aggregate (MTA) and four concentrations of simvastatin/MTA in combination. The study was conducted in two stages using four different simvastatin concentrations and MTA as a capping material for rat maxillary molars. The grades of inflammation and continuity of dentin formation were evaluated in hematoxylin and eosin (HE)-stained samples. Dentin thickness was determined by histomorphometric analysis, and the data were subjected to statistical analysis. On day 3, mild inflammation was observed in all groups. On day 7, the simvastatin groups showed a slightly higher rate of chronic inflammation. Inflammation was not present on day 30. Discontinuous dentin was present in all methylcellulose (control) samples. Continuous dentin was formed in all of the samples treated with 1.5% simvastatin. The greatest dentin thickness was observed after treatment with 1.5% simvastatin and MTA, followed by 0.5% simvastatin. Statistical analysis demonstrated no significant differences in dentin thickness and continuity between MTA and simvastatin at 0.5% and 1.5% (P > 0.05).

Keywords: simvastatin; MTA; direct pulp capping; rat molars; histomorphometry.

Introduction
Direct pulp capping (DPC) is a treatment whereby biocompatible agents are placed over vital exposed pulp to seal and protect it against bacterial penetration (1,2). The outcome of successful direct pulp capping is preservation of pulpal tissues and dentin bridge formation (3). Recently, attempts have been made to develop a cost-effective and safe pulp capping material that biologically activates odontoblasts and promotes dentinogenesis of dental pulp cells (4).

Simvastatin is a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor used as a cholesterol-lowering drug. Its long-term use worldwide has confirmed that it can be used as a low-cost drug with high safety. It is known to have pleiotropic effects including induction of angiogenesis and bone formation and anti-inflammatory properties (5-7). It is also known to regulate the survival of neuronal cells and increase neurogenesis (8). Previous
in vitro experiments have shown that dental pulp stem cells (DPSC) treated with simvastatin at 1 µmol/L showed increased expression of angiogenesis growth factors and odontoblastic differentiation markers, as well as enhanced ALP activity and formation of mineralized nodules (9-12). A recent animal study also found improvement of DPSC-induced pulp regeneration in pulpotomized teeth after treatment with simvastatin at 1 µmol/L (13). Together, these findings suggest that simvastatin might be an effective pulp capping material. However, taking into account that high concentrations of this drug have resulted in increased rates of cell death (9,14), the use of simvastatin as a pulp capping material requires thorough evaluation of the optimum dose.

Mineral trioxide aggregate (MTA) is a bioactive material showing reliable results for pulp capping. MTA promotes differentiation of pulpal cells to odontoblast-like cells and increases the secretion of angiogenic factors (15). Studies have indicated a greater frequency of dentin bridge formation and a lower degree of pulpal inflammation when MTA is used as a pulp capping agent in comparison to calcium hydroxide (16). MTA produces a high alkaline pH and induces hard tissue formation. Previous investigations of the physical properties of MTA have demonstrated good marginal adaptation, sealing ability (17,18) and low or no solubility (19).

Based on previous in vitro investigations of simvastatin, the present study was designed to histologically compare the results of direct pulp capping with MTA and four different concentrations of simvastatin/MTA in rat molars as pulp capping materials, using time periods of 3, 7 and 30 days.

Materials and Methods

All the procedures in this animal study were approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (Tehran, Iran) with the registration no. 145. All efforts were made to reduce animal suffering.

Gel preparation

Methylcellulose gel (MC) served as the simvastatin (Sim) delivery system in this study. A 3% MC gel was prepared by adding the required amount of powder to hot distilled water and cooling it down at room temperature for 24 h. Afterwards, the simvastatin powder was weighed and dissolved in 100% ethanol and added to the previously prepared MC to obtain a homogeneous gel. In order to produce 0.5%, 1.5%, 3%, and 5% Sim gel, 0.5 mg, 1.5 mg, 3 mg, and 5 mg of Sim were added to 100 µL of MC (20,21).

Stage 1 (pilot stage)

Sample assignments

Forty-five male Wistar rats aged 8-9 weeks and weighing 300-400 g were used in this experimental study. The animals were provided with adequate food and water, and kept in plastic cages. A total of 90 sound first maxillary molars were used as samples. Groups were designed as belows: 1. Sim 0.5%/MTA (n = 15), 2. Sim 1.5%/MTA (n = 15), 3. Sim 3%/MTA (n = 15), 4. Sim 5%/MTA (n = 15), 5. MC/MTA (n = 15), and 6. MTA (n = 15).

The animals were assigned to the different groups randomly. Each group consisted of five teeth for time periods of 3, 7, and 30 days. The MC group served as the control and MTA was used as the standard material for DPC.

Surgical procedure

For anesthesia, 100 mg/kg ketamine hydrochloride 10% (Alfasan, Woerden, The Netherlands) and 20 mg/kg xylazine 2% (Alfasan) were injected intraperitoneally after each rat had been weighed.

The rats were fixed on the operating board and the mouth was kept open with orthodontic wires during the operation. Because of the small size of rat molar teeth, use of a rubber dam was not possible. The teeth were mechanically cleaned with a micro-brush and the oral cavity was irrigated with chlorhexidine gluconate 0.2% (Iran Najo Pharmaceutical Co., Tehran, Iran). With the aid of magnifying glasses (2.5×, Heine, Hersching, Germany), cavities were prepared on the occlusal surface of the right and left first maxillary molars with sterile cylindrical diamond burs #010 (Teekskavan Inc., Tehran, Iran) in a high-speed handpiece (Midwest Dental Products Corp., Des Plaines, IL, USA). Cavities were prepared under permanent cooling with water spray. Afterwards, the roof of the pulp chamber was exposed using a sterile sharp probe (Dentsply-Maillefer, Ballaigues, Switzerland). Bleeding was controlled by applying 2.5% NaOCl, and a sterile cotton pellet was pressed onto the exposed site. The cavity was rinsed with normal saline solution and then dried using sterile cotton pellets.

The Sim or MC gels were placed on the exposed sites in each group with a sterile dycal applicator to cap the pulp. White MTA (Angelus, Londrina, Brazil) was prepared with a powder to distilled water ratio of 3:1, as recommended by the manufacturer. For the next step, a thin layer of MTA was gently placed on the gel using the MTA carrier. In group 6, MTA was directly placed on the exposed site as the capping material.

A two-step self-etching bonding agent (Clearfil SE Bond, Kuraray Inc., Okayama, Japan) was used.
The primer was applied with a microbrush, and after 20 s dried with a mild air flow in accordance with the manufacturer’s instructions. The bonding agent was then applied to the surface with the microbrush, and after a gentle air flow was light-cured for 10 s. The cavity was filled with flowable composite resin A2 (Opallis, FGM Dental Products, Joinville, Brazil) and cured for 20 s.

Slight occlusal reduction of the first mandibular molars was performed to reduce the masticatory force on the upper molars. After the operation, the rats were fed with a soft diet.

**Tissue preparation and serial sectioning**

The animals were sacrificed by an overdose of ketamine hydrochloride at 3, 7, and 30 days after pulp capping. Five teeth at each time interval were assigned to each group. The maxillae were separated and kept in 10% buffered formalin solution for 2 days. The specimens were decalcified in 10% formic acid for 7-10 days at room temperature, and then trimmed of excess tissues. The filling materials remaining were then removed carefully from the cavity, and the specimens were prepared for hematoxylin and eosin staining. Serial sections 4 µm thick were cut longitudinally near the exposure site and stained with hematoxylin-eosin. The sections were evaluated using a light microscope (Eclipse E 400, Nikon Co., Tokyo, Japan) at magnifications of ×40, ×100, ×200, and ×400 by a blinded oral and maxillofacial pathologist.

**Evaluation criteria**

**Inflammatory cell infiltration**

The number of inflammatory cells among 100 pulpal cells close to the defect was counted and reported as listed below for each of the 3-, 7-, and 30-day groups (22,23). Score 0: 0-10%, Score 1: 10-30%, Score 2: 30-50%, and Score 3: >50%. Necrotic debris under the pulp capping material was not considered for inflammatory cell evaluation. The number of inflammatory cells in the pulp chamber was counted.

**Dentin bridge formation and histomorphometric analysis**

Bridge formation was evaluated in different pulp capping groups after 30 days. Continuous or discontinuous dentin formation at the exposure site was recorded. A minimum number of three sections were obtained from each sample, and images were taken at a magnification of ×100 with a digital camera (E8400, Nikon Co.). The thickness of the dentin formed was measured by histomorphometric analysis using Iranian Histomorphometric Software (Version 2.0, SBMU, Tehran, Iran). The mean value of dentin thickness in these sections was recorded as the final value for dentin formation (24).

**Statistical analysis**

The data were analyzed using SPSS 18.0 (SPSS Inc, Chicago, IL, USA). Chi-squared and Fisher’s Exact tests were used to compare the continuity of dentin formation and inflammation. The descriptive values consisting of mean and standard error were assessed for dentin thickness in each group. Homogeneity of variance was tested using the Levene Statistic, followed by the Kruskal-Wallis and Mann-Whitney tests for paired comparisons of dentin thickness between groups. The level of confidence was set at 0.05.

**Stage II**

Based upon the results obtained from the pilot stage, two concentrations—0.5% and 1.5%—were selected for this stage. A total of 15 Wistar rats (30 maxillary molar teeth) were used based on the same criteria as the pilot study. The animals were randomly assigned to the following three groups: Sim 0.5%+MTA (n = 10), Sim 1.5%/MTA (n = 10), and MTA (n = 10). All the procedures were performed as defined for the pilot stage. The rats were sacrificed on day 30, and after sample preparation, histologic evaluations were performed according to the criteria mentioned previously.

**Results**

**Stage I (Pilot stage)**

**Inflammation**

Inflammation scores 2 and 3 were not observed during the entire experimental period. On days 3 and 7, the samples showed inflammation scores of 0 and 1 in the different groups. On day 30, inflammatory cell infiltration was absent in all of the samples.

The distribution of inflammatory cells on days 3 and 7 is demonstrated in Table 1. On day 7, the simvastatin groups showed a higher rate of chronic inflammation regardless of the simvastatin dose. However, Fisher’s Exact test showed no significant difference between

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**Table 1** Number of samples with inflammation scores of 0 and 1 on days 3 and 7 in the different groups

|        | Score 0 | Score 1 | Score 0 | Score 1 |
|--------|---------|---------|---------|---------|
| Day 3  |         |         |         |         |
| Sim 0.5% + MTA | 5     | 0      | 4       | 1       |
| Sim 1.5% + MTA | 5     | 0      | 4       | 1       |
| Sim 3% + MTA  | 3      | 2      | 4       | 1       |
| Sim 5% + MTA  | 5      | 0      | 4       | 1       |
| MC + MTA     | 5      | 0      | 5       | 0       |
| MTA          | 4      | 1      | 5       | 0       |
Infiltration of inflammatory cells in the Sim 1.5\% group is presented in Fig. 1.

Dentin formation
In all samples, dentin formation was observed on day 30. HE samples in each group are presented in Fig. 2.

Dentin continuity
Continuous dentin formation was not observed in the MC group. In the Sim 0.5\% group, continuous dentin was present in all of the samples except one. In all other samples, a continuous dentin bridge completely covered the exposure sites. As an intact dentin barrier was not present in any of the samples in the MC group (control), chi-squared and Fisher’s Exact test showed a significant difference in the continuity of dentin between the groups ($P < 0.05$), while a continuous dentin barrier was observed in all other samples with the exception of one in the Sim 0.5\% group. Comparison between the Sim 0.5\% group and other experimental groups showed no significant difference ($P = 1.00$). However, there was a statistically significant difference between the Sim 0.5\% and MC groups ($P = 0.04$).
Dentin thickness

The MC (control) group showed the lowest dentin formation with a thickness of 23 ± 3.4 µm. The highest range of dentin thickness was obtained with Sim 1.5% (152 ± 43.5 µm) and MTA (150 ± 29.7 µm). The Sim 0.5% group also showed a thick dentin barrier (110 ± 18.4 µm), while dentin formation in the Sim 3% (67 ± 4.8 µm) and Sim 5% (54 ± 6.2 µm) groups was lower.

Kruskal-Wallis test demonstrated a significant difference between the study groups (P = 0.00). For comparison of two groups in a paired manner, Mann-Whitney U test was used. There were no significant differences between the MTA and Sim 0.5% (P = 0.31), MTA and Sim 1.5% (P = 0.15), Sim 0.5% and Sim 1.5% (P = 0.69), and Sim 3% and Sim 5% groups (P = 0.14) groups. Paired comparison of MC with all the experimental groups showed a significant difference (P < 0.05). In addition, there were significant differences between the Sim 0.5% and Sim 3% (P = 0.015), Sim 0.5% and Sim 5% (P = 0.008), Sim 1.5% and Sim 3% (P = 0.032), Sim 1.5% and Sim 5% (P = 0.016), MTA and Sim 3% (P = 0.016), and MTA and Sim 5% (P = 0.008) groups.

Overall results: stages I and II

The data for dentin thickness and continuity in 15 samples from the pilot stage and stage II, in each of the Sim 0.5%, Sim 1.5% and MTA groups at the 30-day time point are presented below.

Inflammation

All the samples showed grade 0 inflammation at this stage. Therefore, no statistical analysis was performed.

Dentin continuity

In the Sim 0.5% group, three discontinuous dentin bridges were formed among all the samples, and the MTA group showed only two discontinuous dentin bridges. However, in all of the samples in the Sim 1.5% group, continuous dentin bridges were observed. The chi-squared test demonstrated no significant differences among these groups (P = 0.20).

Dentin thickness

Dentin thickness was maximal in the Sim 1.5% group (156.3 ± 25.9 µm) and MTA group (154.6 ± 16.8 µm), followed by the Sim 0.5% group (120.6 ± 15.3 µm) (Fig. 2). Kruskal-Wallis analysis showed no significant differences between the groups (P = 0.35).

Discussion

In this animal study, rat maxillary molars were used as miniature models of human molar teeth for direct pulp capping, due to their anatomical, biological and histological similarity, as well as the process of pulpal repair (25). Direct pulp capping investigations of animal teeth have usually focused on infiltration of inflammatory cells, pulp preservation, bacterial penetration and hard tissue formation (18,23). Evaluation criteria have also included the quality of dentin formed (reparative/reactive) and its continuity and location. Dentin thickness has also been measured in some studies using histomorphometric analysis (24,26).

The present study was designed to assess the most efficient doses of simvastatin—a safe and low-cost drug—as a direct pulp capping agent, and to histologically compare the results obtained with those for MTA as a standard pulp capping material. However, the long-term sealing properties and solubility of simvastatin gel may be a concern. To overcome this issue, in the simvastatin and MC groups a layer of MTA was placed on the gel (27). Glass ionomer cement was not applied for covering the gel, due to its early moisture sensitivity (28) and possible irritation of pulpal tissue (27). A pilot study was first conducted to determine the preferable concentrations of simvastatin for this procedure. The next stage was then designed to allow a stronger conclusion about the efficiency of this drug to be reached, by increasing the number of samples.

The inflammation rates on days 3 and 7 in the study groups were not significantly different. However, on day 7, all of the simvastatin groups showed a slightly higher rate of chronic inflammation. This can be considered a pulpal response to simvastatin. It can also be speculated that due to the mild to moderate rate of inflammation in the simvastatin groups, the process of pulpal repair and angiogenesis may have been further facilitated through release of inflammatory cytokines and mediators (29). Nevertheless, on day 30, no inflammation was observed in the stage I and stage II samples.

In this study, thickness and the continuity of the dentin formed were the criteria used to indicate the rate of odontoblast-like differentiation in the samples (30). For more accurate evaluation of Simvastatin efficiency, it is suggested to use immunohistological staining to assess odontoblastic activity and the nature of the hard tissue formed (31). Micro-CT analysis is also suggested for accurate measurement of dentin thickness. In the control (MC) group, discontinuous dentin bridges were observed, possibly indicating the absence of odontoblast-like differentiation. Histomorphometric analysis showed that the mean thicknesses of the dentin bridges was greater in the Sim 1.5% and MTA groups and relatively
lower in the Sim 0.5% group at both stages. However, there was no statistically significant difference between these three groups, possibly due to the small sample size. On the other hand, the thickness of the dentin formed at other simvastatin concentrations and in the MC group was significantly lower (groups 3, 4, and 5). These results reveal that simvastatin is efficient as a capping material only at specific doses; higher concentrations may be toxic to cells. Methylcellulose used in the control group would also have adversely influenced the efficiency of MTA. Lack of an efficient pulp capping material and absence of MTA in direct contact with the vital pulp would explain the result obtained in the control group (32). The amounts of dentin formed under each of the pulp capping materials at stages I and II were within the same range. At stage II, discontinuous dentin formation was also observed in MTA samples other than the Sim 0.5% group, whereas all the Sim 1.5% samples showed continuous dentin formation. Therefore, only Sim at 1.5% facilitated continuous dentin formation in all samples at both stages. This will need to be further evaluated using larger sample sizes.

Two previous clinical studies have used simvastatin as a direct pulp capping material (33,34). Aslaminabadi et al. (33) compared the dentin formation and inflammation rates after direct pulp capping with simvastatin gel at concentrations of 1, 5, and 10 µmol with calcium hydroxide, and found higher rates of complete dentin formation in the calcium hydroxide group. Complete dentin formation was reduced when higher simvastatin concentrations were used. The inflammation and necrosis rates became higher as the simvastatin concentration increased. In another clinical study involving clinical and radiographic examinations at 2, 6, and 12 months after treatment, Aslaminabadi et al. (34) found that simvastatin in combination with a triple antibiotic paste (ciprofloxacin, cefixime, metronidazole) showed an efficacy similar to that of MTA as a direct pulp capping agent.

As the dose-dependent effects of simvastatin on human pulp were confirmed in the previous clinical study (33), it seemed necessary to evaluate the optimum concentration of simvastatin in animal experiments. The 0.5% and 1.5% concentrations used in the present animal study yielded the best results. Our findings also suggested that the effects of simvastatin at concentrations between 0.5% and 1.5% would be worth investigating.

Simvastatin has been used previously in combination with dental cements, such as calcium phosphate cement and α-tricalcium phosphate (TCP) (4,35,36). In an in vitro study, the effects of simvastatin combined with TCP on human dental pulp cells were compared with those of MTA and TCP (4). Cells treated with TCP + Sim showed higher cell growth and ALP activity than those treated with MTA. The levels of mineralization markers in the TCP + Sim group were also comparable to those in the MTA group. Therefore, we hypothesize that addition of simvastatin at an appropriate dose to MTA powder may improve its efficiency for pulp capping procedures. Meanwhile, the effects of simvastatin on the properties of cement, such as the setting time, and compressive and tensile strength, must be taken into consideration.

It can be concluded that simvastatin gel at a concentration of 1.5% under MTA might promote active pulpal repair when applied as a direct pulp capping agent, considering that high-quality dentin was continuously formed in all of the samples treated with this material. Further studies with a larger sample size will be needed to confirm the present results, and also to investigate the differentiation of odontoblast-like cells in simvastatin-treated samples.

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