



REGENERATIVE POTENTIAL OF SIMVASTATIN ON DENTAL PULP AND ADIPOSE-DERIVED STEM IN IMMATURE DOG'S TEETH: A HISTOLOGIC AND RADIOGRAPHIC STUDY

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ABSTRACT

Objective: This study was directed to evaluate the regenerative potential of simvastatin (SIM) on dental pulp stem cells (DPCSs) and Adipose-derived stem cells (ASCs) in non-vital immature dogs' teeth both histologically and radiographically. **Methods:** The present study was carried out on 6 adult healthy male mongrel dogs aged from 4 to 6 months and their weight ranged from 12 to 17 kg. Three premolars (two rooted) in each quadrant were included in this study summing up the total number of teeth to 72. Endodontic access cavities were prepared in all experimental teeth in the buccal surface and the vital pulps of teeth were exposed till confirmation of radiolucency. Then triple antibiotic paste was used for disinfection of root canals for 3 weeks. After disinfection period, the teeth were divided into two groups: group 1; treated with dental pulp stem cells and simvastatin; while group 2 was treated with adipose-derived stem cells and simvastatin. Both groups were evaluated histologically and radiographically after 1 week, 1 month and 3 months. **Results:** Better radiographic and histologic findings were found in the group treated with dental pulp stem cells with simvastatin than groups treated with adipose-derived stem cells and simvastatin after all evaluation periods. **Conclusions:** Under the condition of this study, simvastatin aids in regeneration of infected immature dog's teeth with the ability of producing functional dentin-pulp complex and inclusion of dental pulp stem cells seemed to improve the outcomes of attempted regeneration.

Key words: Simvastatin, Dental pulp stem cells, Adipose-derived Stem Cells, Immature dog's teeth, Regenerative Endodontics.

INTRODUCTION

Treatment of immature teeth with necrotic pulps has been considered a big challenge because interruption in root development leads to weak and thin fragile dentinal walls liable to fracture, beside the difficulty to achieve an adequate apical seal using conventional root canal filling techniques. Traditional management of such teeth ranges from induction of apical closure using calcium hydroxide (Apexification), apical plug using bioactive materials such as MTA, to apical surgery with retrograde sealing⁽¹⁾. Recently,

regenerative endodontics has gained attention as a biologically based alternative as it can allow for further root maturation in length and thickness by the regenerated vital tissue. With advancement of regenerative protocols, it is now possible to use stem cells for dental tissue engineering either from dental or non-dental origin⁽²⁾. Alternatively, different pharmacologic agents have been introduced to allow for enhancing this regenerative protocol. Recently, Simvastatin (SIM)-treated stem cells exhibited enhanced odontogenic differentiation and accelerated mineralized tissue formation. This observation led to the possibility of utilizing

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Dental Pulp Stem Cells (DPSCs) in combination with simvastatin to regenerate pulp and dentin for clinical applications⁽³⁾. From the histological point of view, the nature of the tissue formed in the canal space in human revascularized immature permanent teeth is speculative because no histologic studies are available⁽⁴⁾. Therefore, animal models may shed some light to address this issue. The combination of simvastatin with stem cells from different sources to restore a functional dentin-pulp complex is not fully investigated in dental literature.

MATERIALS AND METHODS

The procedures were carried out in the Department of Veterinary Surgery, General veterinary hospital in Alabbasyya, Cairo, Egypt. Six adult healthy male mongrel dogs aged from 4 to 6 months and their weight ranged from 12 to 17 kg were used in this study. The dogs were examined to rule out the presence of any disease. dogs were vaccinated by the triple vaccine against rabies, distemper, leptospirosis and hepatitis viruses. Three premolars (two rooted) in each quadrant were included in this study summing up the total number of teeth to 72 (12 premolars x 6 dogs). In the first visit, endodontic access cavities were prepared in all experimental teeth in the buccal surface with high-speed hand piece (NSK, Tokyo, Japan) under constant coolant with a size no. 2 sterile carbide bur (Brassler USA, Savannah, Georgia). Then a size 25 sterile k-file (Mani, Inc., Tochigi, Japan) was used to disrupt the pulp tissue in the canals followed by placement of a piece of cotton into the entrance of each canal.

In the second visit, rubber dam isolation of the selected teeth was done and root canals were irrigated using 10 mL of 1.5 % sodium hypochlorite (Regular household Bleach, Clorox, Egypt), flushed with 10 ml of sterile saline (0.9% sodium chloride, Novartis, Egypt) and dried with sterile paper points. The triple antibiotic paste (TAP) was prepared using metronidazole 500 mg tablets (Flagyl 500

mg, Aventis, Cairo, Egypt), ciprofloxacin 250 mg tablets (Ciprocin 250 mg, EPICO, Cairo, Egypt) and doxycycline 100 mg capsules (Vibramycin, Pfizer, Cairo, Egypt) in equal portions of each antibiotic by concentration mixed with 3-4 drops of sterile saline to a paste like consistency. TAP was injected into the canal using a 16-gauge needle and the access cavities were sealed with a resin reinforced glass ionomer. After disinfection period (3 weeks), antibiotic paste was removed by copious irrigation using 10 mL of 1.5 % sodium hypochlorite then flushed with normal saline using a 27-gauge side vented closed end endodontic irrigating needle. A 17% EDTA liquid was used as final rinse then the root canals were dried using paper points.

According to sample size calculation, the number of selected teeth included within the study was set to be 72 teeth included in 6 dogs. Teeth were divided into two equal groups according to different treatment protocols used for each experimental group as follows: Group 1: treated with Dental Pulp Stem Cells (DPSCs) and simvastatin (SIM) hydrogel and Group 2: treated with Adipose Stem Cells (ASCs) and simvastatin (SIM) hydrogel.

Then each main group was subdivided into 3 subgroups according to post treatment evaluation periods as follows: subgroup 1: evaluation period after 1 week of treatment, subgroup 2: evaluation period after 1 month of treatment and subgroup 3: evaluation period after 3 months of treatment. Extraction of maxillary lateral incisor with vital pulp was done from each dog to isolate dental pulp stem cells. A longitudinal inciso-apical groove and a vertical split were done while the pulp was exposed and picked up using toothed forceps. The harvested pulp was placed in a minimum essential medium (MEM) transporting media (Sigma Aldrich, St. Louis, MO, USA) in a validated, temperature-controlled 2°C transport box fitted with a frozen cold pack and shipped to the stem cells laboratory for processing.

The harvested dental pulp was removed from the transport media, washed in Phosphate buffered Saline (PBS) to ensure removal of any exogenous debris, placed in a sterile petri dishes and cut into small pieces using a sterile scalpel blade. On the other hand, autologous adipose tissue was harvested from the pad of fat in the abdominal region through an elliptical incision in the skin till reaching the fatty layer. Injection of 1:50:000 epinephrine into the adipose compartment was made to minimize blood loss and to decrease blood flow at the surgical site.

Then adipose tissue was resected using size 15 surgical scalpel. Thereafter, the harvested adipose tissue was placed in a minimum essential medium (MEM) transporting media and shipped to the stem cells laboratory for processing in the same way of pulp tissue. Enzymatic digestion of tissue was done as described by Gronthos et al. ⁽¹⁴⁾ where 3 mg/ml collagenase type I were used as digesting solution. The harvested tissues were then incubated at 37°C in a humidified atmosphere containing 5% CO₂ for the time necessary to allow cells to slip down from the explants.

After incubation, the digestive reaction was stopped by the addition of fresh basic medium to neutralize collagenase. The tubes were centrifuged for five minutes at room temperature to concentrate cells at the bottom of the tubes. A sterile serological pipette was used to transfer the medium containing cells into T-25 cell culture flask (Falcon, Franklin Lakes, NJ) with filter top cap. The flask was then incubated at 37°C in a humidified atmosphere containing 5% CO₂ for the time necessary for adherence. After washing the cells with Hanks Balanced Salt Solution (HBSS), 1 ml of Trypsin was added to the flasks in non- cell side to detach the cells from the flask floor (trypsinization). Then 5 ml of medium was added to the flask and trypsin/medium/cell suspension had been collected in 15 ml test tube for centrifuging at 600 rpm for 6 minutes. Suspension was vacuumed out to get out as close

as possible to the pellet without touching the cells. Then splitting of the cell suspension was done equally in T 25 flasks and the flasks were put on their sides in the incubator with loose caps.

After 7 days, the cells were observed under microscope and non-adherent cells were discarded. In these experimental conditions the cells reached confluence within 15-20 days of culturing through three passages. Counting of the cells was done using hemocytometer and Trypan blue stain was used to detect viable cell count. Simvastatin was dissolved in absolute ethanol (50 mg: 1 ml) and mixed with a magnetic stirrer then stored at 4°C for future use. The selected dose was 0.1µl/L of simvastatin. Methylcellulose gel (MC) 4.0% w/v (Guangzhou Qiyun Biotechnology, China) served as the simvastatin delivery system after dissolving in hot triple-distilled. DPSCs were transplanted as described by Krebsbach et al., 1997 ⁽⁵⁾ and exposed to 0.1 µml/L simvastatin hydrogel ⁽⁶⁾.

After each evaluation period, the dogs were sacrificed under general anesthesia provided by pentobarbital where The carotid arteries were exposed and cannulated then the dogs were euthanized with additional pentobarbital at a dose of 90 mg/kg IV. The animals were perfused with 10% buffered formalin (Fisher Scientific, Fair Lawn, New Jersey). After euthanasia of the dogs, the maxillary and mandibular jaws were surgically removed and divided at the midline into two halves right and left one using a bone saw. The jaws with the involved teeth were decalcified, dehydrated and sections were made longitudinally every 5 µm through the apical foramen of the roots. Tissues were stained with Hematoxylin and Eosin (H&E) and evaluated under light microscopy at up to 400 X magnification for the presence or absence of the newly formed tissues. Each individual root was taken as a unit of measurement/assessment and graded for the inflammatory changes, intracanal tissue changes, hard tissue formation and apical closure. Statistical analysis was performed

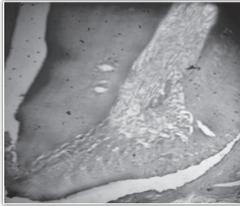
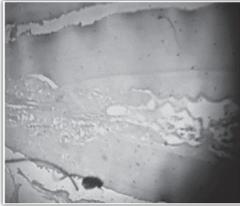
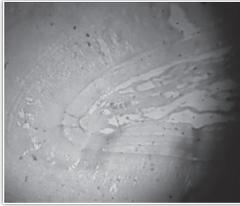
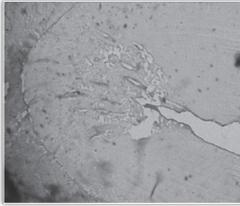
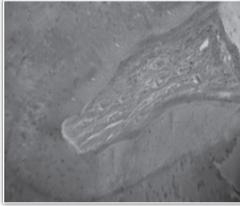
with IBM® SPSS® Statistics Version (Statistical Packages for the Social Sciences 20, IBM, Armonk, NY, USA). Data were represented by total number and percentage for each method used in the study. Descriptive statistics was done using one way ANOVA and Pearson correlation (r) tests for comparing the relation between different groups. Significance was set at P value ≤ 0.05 .

RESULTS

Histological comparison among the tested groups after different evaluation periods

- **Inflammatory changes:** For both groups there was a statistically significant inflammatory changes between different periods one weak, one month and 3 months as p= 0.003, 0.003, 0.028 and 0.003.
- **Intracanal tissue changes:** For both groups at one weak, one month and 3 months, there was a statistically significant difference as p= 0.019 and 0.007 respectively. DPSCs+SIM recorded the better change.
- **Hard tissue changes:** For both groups, there was a statistically significant changes between different periods one weak, one month and 3 months as p= 0.00 and 0.001 respectively. For one month and 3 months, there was no a statistically significant difference as p=0.34.and p=0.34.
- **Apical closure:** For DPSCs+SIM, there was a statistically significant difference between one weak, month and three months as p=0.05. Better apical closure was recorded at 3 months followed by one month. For ASCs+Sim, there was a statistically significant difference between one weak, month and three months as p=0.00. Better apical closure was recorded at 3 months followed by one month. For 3 months, there was a statistically significant difference between both groups as p=0.0019. DPSCs+Sim showed the better apical closure followed by ASCs+SIM.

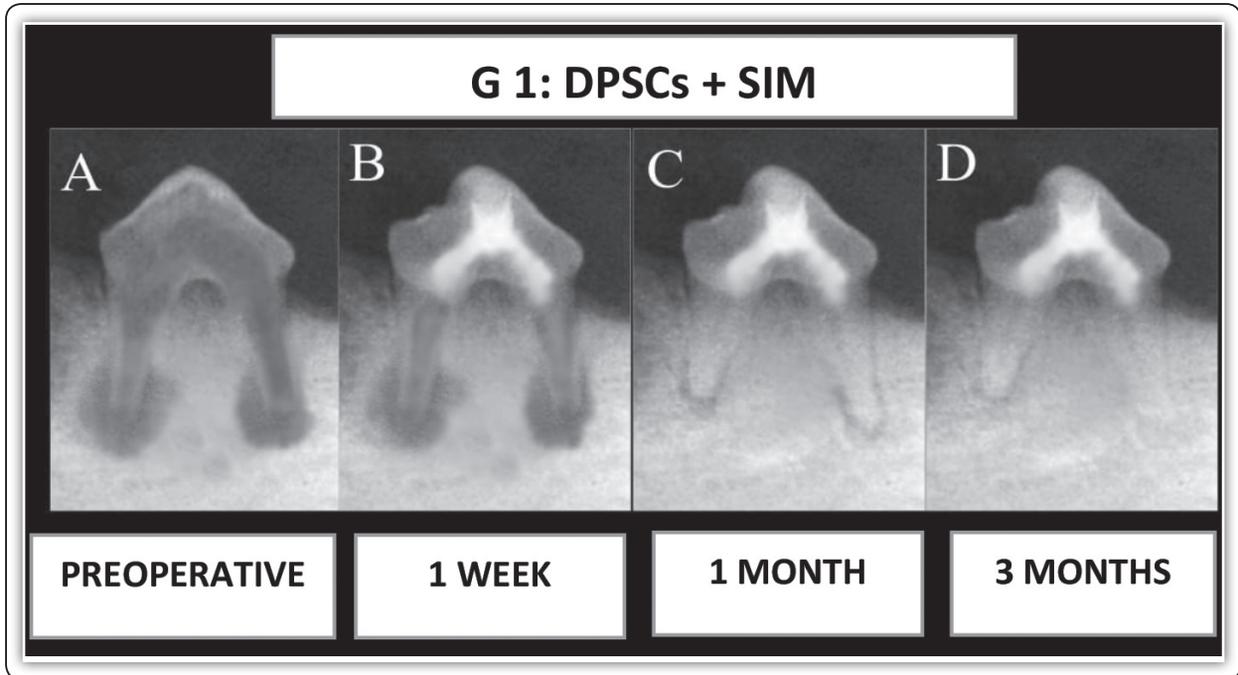
TABLE (1): Showing histological sections of the four groups over different evaluation periods:

Groups	DPSCs+SIM	ASCs+SIM
1 w		
1 m		
3 m		

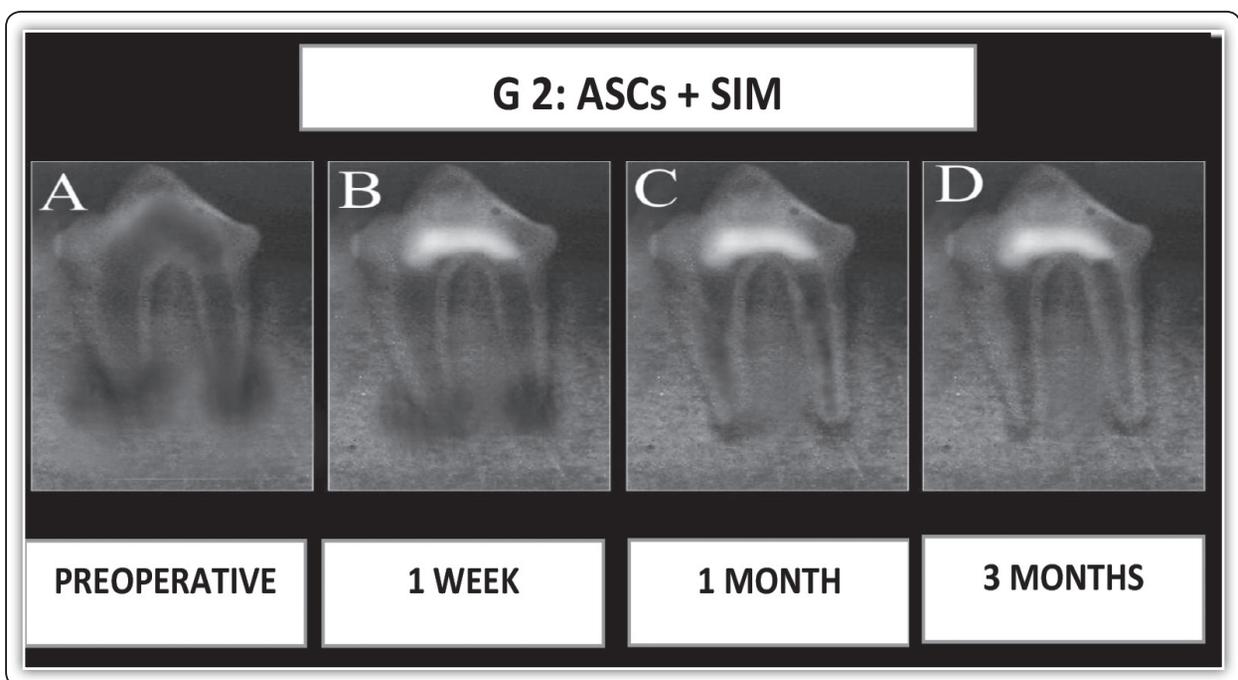
Evaluation of the radiographic findings:

- **Radiolucency:** For DPSCs+SIM, there was a statistically significant difference between one week, one month and three months. Better radiolucency decrease was recorded at 3 months followed by one month. For ASCs+SIM, there was a statistically significant difference between one week, month and three months. Better radiolucency decrease was recorded at 3 months followed by one month.
- **Apical closure:** For DPSCs+SIM, there was a statistically significant difference between one week, one month and three months. Better apical closure was recorded at 3 months and one month followed by one week. For ASCs+SIM, there was no statistically significant difference between one week, one month and three months.

- Canal calcification: For DPSCs+SIM, there was a statistically significant difference between one week, one month and three months. canal calcification was significant at 3 months and one month when compared with one week. For ASCs+SIM, there was no statistically significant difference between one week, one month and three months.



Radiographic changes of group 1 over different evaluation periods



Radiographic changes of group 2 over different evaluation periods

DISCUSSION

Treatment of immature teeth with necrotic pulps represents a big challenge because interruption in root development leads to weak and thin fragile dentinal walls with difficulty to achieve an adequate apical seal using conventional techniques. Recently, regenerative endodontics has gained attention as a biologically based alternative as it can allow for further root maturation by the regenerated vital tissue⁽⁷⁾. The choice of dogs as an animal model for biological experiments in endodontics is based on the fact that they have similar apical repair compared with humans but over a short duration (average one-sixth of human) due to the high growth rate. Also, dogs have a close similarity in radicular structure to immature human teeth in their open apex characteristics⁽⁸⁾. The age of the dogs ranged between 4 and 6 months which was suitable for the study of immature teeth, as premolar teeth are immature at this age range.

With regards to root canal disinfection, a concentration of 1.5% of NaOCL was used as it has the least cytotoxic effect on the stem cells compared to other concentrations⁽⁹⁾. Final irrigation was done by 17% EDTA in order to expose growth factors entrapped in the dentin matrix including, bone morphogenic protein 2 (BMP2), Transferring Growth Factor-B (TGF-B) and angiogenic factors as platelets-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF 2)⁽¹⁰⁾. With regards to the coronal seal, MTA was used in the coronal part of the root canal because it is a bioactive material that has an excellent sealing property and considered the golden standard in this type of research⁽¹¹⁾. DPSCs were used in this study due to their advantages in clinical usage including lower mortality rate, less legal or ethical issues, easy access from extracted teeth, and cryopreservation without losing their multi-differentiation potential.

Another type of non-dental adult stem cells used was Adipose-Derived Stem Cells (ASCs) which

have several advantages including ease of isolation by local excision or suction-assisted liposuction, relative abundance, rapidity of expansion, high cell yield, rapid in vitro expansion and broad multipotency with differentiation into number of cells lineages including adipo-, osteo-, chondrocytic lineages that is independent upon serum source and quality⁽¹²⁾.

Enzymatic digestion method was used for isolation or as it is widely used in laboratories and preferable to detect, isolate, proliferate and differentiate stem cells⁽¹³⁾. Simvastatin (SIM) was used due to its high bone growth stimulation capability compared with the other hydrophilic Statins and its anti-inflammatory effect when delivered or applied locally. It is becoming increasingly evident that the ideal scaffold for dental pulp tissue engineering will be injectable, not casted. This is because of the narrow spaces within the root canal and the complexity of its anatomy, particularly in the apical region so hydrogels can penetrate throughout the root canal system⁽¹⁴⁾.

Regarding inflammatory changes of the tested groups, the results showed initial increasing followed by marked reduction along the different evaluation periods. This indicates the progression of healing of periapical lesion and reduction of inflammatory reaction with time. This is in agreement with the findings of inflammatory reaction by wang et al⁽¹⁵⁾ and Pereira MS et al⁽¹⁶⁾ who advocated the presence of mild inflammatory reaction as it may provide factors to guide the differentiation of stem/progenitor cells in the healing soft tissue into cementoblasts. On the other hand, the reduction of inflammation was an indication of progressed healing of the periapical lesion over time that is in agreement with Gomes-Filho et al. 2011⁽¹⁷⁾.

This finding was also in agreement with Lu D et al⁽¹⁸⁾ who revealed that, statins have an anti-inflammatory effect in various tissues and this could help restore the inflamed pulp tissue beside their ability to accelerate reparative dentin formation.

Regarding intracanal tissue changes of the tested groups, the results showed that DPSCs+SIM group has the highest change. This may be attributed to the power of DPSCs as a native progenitor cells that have odontogenic rather than osteogenic development⁽¹⁹⁾.

Adversely, ASCs+SIM group showed the least intracanal tissue changes that might be due to that ASCs had a little effect on pulp regeneration unlike pulp cells that are the progenitor of DPSCs⁽²⁰⁾. This is in agreement with other study demonstrated the proliferative ability and developmental potentials of DPSCs beside their ability to develop into distinct tissues representative of the microenvironments from which they were derived *in vivo*. Also, the potent neurogenicity of dental stem cells may be attributed to their neural crest origin⁽²¹⁾. Also SIM has a high regenerative potential effect on DPSCs that support the finding of the present study. Regarding ASCs+SIM, the results of the present study were in disagreement of another investigation demonstrated that transplantations of bone marrow-derived stem cells and adipose-derived stem cells can induce pulp regeneration in the root canal after pulpectomy in dogs.

The information about the hard tissue produced on dentinal walls and the cells responsible for hard-tissue production is still lacking. The results of this study was in agreement with a study done by Batouli et al.⁽²²⁾ showed that when DPSCs are seeded onto human dentin surfaces and implanted into immunocompromised mice, reparative dentin-like structure is deposited on the dentin surface. Also some reports done by Laino et al.,⁽²³⁾ showed that DPSCs have osteogenic potential and may form bone-like structure *in vitro* and *in vivo*. After one-month evaluation period, DPSCs+SIM group showed that the degenerative alterations gradually resolved. A replacement by regenerative tissues began to appear through a regular arrangement of the pulp tissue architecture. Later on, an improvement of the pulp tissue architecture mimicking the normal

pattern was seen after 3 months' evaluation period.

With regard to the changes in apical closure of the tested groups, better apical closure recorded for DPSCs+ SIM at 3 months indicating the possible effectiveness of this combination in pulp regeneration along with dentin regeneration. In converse to the present study Hicok et al⁽²⁴⁾ found that ASCs produced more osteoid when being seeded on hydroxyl appetite tricalcium phosphate (HA/TCP) than cells that were cultured on collagen/HA–TCP composite, indicating that the osteogenic capacity of ASCs could be heavily influenced by the scaffold on which cells were seeded. Regarding radiographic evaluation, the results of this study supporting the use of radiographic examination for the confirmation of histologic findings not for accurate measurements. For DPSCs+SIM the highest decrease in periapical radiolucency decrease was recorded at 3 months followed by 1 month.

This may be attributed to the effect of simvastatin that promotes osteoblastic differentiation, enhances ALP production and bone mineralization and up-regulates the expression of bone anabolic factors such as VEGF⁽²⁵⁾. Recent review revealed that SIM can either accelerate or retard mineralized tissue genesis according to the concentration used. At low concentrations, these drugs feature pleiotropic effects with mesenchymal stem cells, increasing the expression of several osteo/odontoblastic markers, such as dentin sialophosphoprotein (DSPP), dentin matrix acidic phosphoprotein 1 (DMP-1), alkaline phosphatase (ALP), collagen type 1 alpha 1 (Col1A1), osteocalcin, osteopontin, runt-related transcription factor 2, and bone morphogenetic protein 2 (BMP-2), leading to intense mineralized matrix deposition *in vitro*⁽²⁶⁾.

Conversely, the higher concentration of SIM may lead to cell death. For DPSCs+SIM canal calcification was significant at 3 months and 1 month when compared with 1 week. The explanation of this finding may also due to the combination of DPSCs+SIM where statin promotes osteoblastic

differentiation, enhances ALP production and bone mineralization, and up-regulates the expression of bone anabolic factors such as VEGF⁽²⁷⁾. Although complete root canal calcification/obliteration is not mentioned as a failure in cases that have undergone regenerative endodontic treatment, it can cause serious challenges in case the involved tooth needs root canal therapy.

CONCLUSIONS

1. Simvastatin aids in regeneration of infected immature dog's teeth with the ability of producing functional dentin-pulp complex.
2. Inclusion of a dental pulp stem cells seemed to improve the outcomes of attempted regeneration.
3. The outcome of regeneration of immature dog's teeth is evident by apical closure with time.
4. Mesenchymal stem cells from different sources are comparable in their regeneration potential in vivo and that their capabilities are independent of their origin

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