

EFFECT OF DEMINERALIZED DENTIN MATRIX GRAFT ALONE AND COMBINED WITH STATIN OR PROPOLIS ON BONE REPAIR IN RABBITS' TIBIA

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ABSTRACT

Background: Demineralized dentin matrix (DDM) is a biologically active tissue could stimulate new bone tissue formation then resorbed during the bone remodeling process. **Aim:** The aim of the present study was to evaluate the effect of demineralized human dentin matrix graft alone and when combined with Statin or Propolis on bone repair in rabbits' tibia.

Materials and Methods: Thirty adult male rabbits (weight 2 to 2.5 kg) were used in this study. In each rabbit four holes were created (two in each tibia), one was left empty as control, the second was packed with DDM slices, the third was packed with DDM slices saturated with statin and the fourth was packed with DDM mixed with propolis. The animals were sacrificed at 2, 4 and 6 weeks postoperatively and the bone specimens were processed for histomorphometric analysis and scanning electron microscopic examination.

Results: Histomorphometric analysis showed significantly high mean bone area for DDM/Statin group followed by DDM group then DDM/Propolis group then control group. SEM findings were supportive to these results.

Conclusions: It was concluded that the DDM was profound and biocompatible material for bone repair. Addition of Statin promotes bone repair process while Propolis retards bone repair especially in early stages.

KEY WORDS: Bone repair, Demineralized Dentin Matrix, Statin, Propolis.

INTRODUCTION

Bone tissue regeneration requires essential factors, such as: osteoprogenitor cells, osteoconductive cells, growth factors, and absence of local infection. It is assumed that the osteoinductive cascade

begins with chemotaxis of bone progenitor cells, angiogenesis, and bone cells differentiation. Bone cell recruitment, division rate, and differentiation are controlled by growth factors, including bone morphogenetic protein (BMP) and transforming growth factor- beta (TGF-beta).⁽¹⁻³⁾ Demineralized

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dentin matrix (DDM) is a biologically active tissue capable of delivering BMP.^(4,5) Recent studies have shown that DDM slices stimulate new bone tissue formation then subjected to resorption during the bone remodeling process.⁽⁴⁻⁹⁾ DDM was also used for dentin regeneration, repair of articular cartilage defects and as carrier material for recombinant human BMP-2.⁽¹⁰⁻¹²⁾

Statins are widely used for lowering cholesterol and the treatment of hyperlipidemia and arteriosclerosis. They are specific competitive inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme.⁽¹³⁻¹⁴⁾ In late 1999, researchers found that statins may have direct effects on bone through their ability to stimulate bone morphogenetic protein-2 (BMP-2) promoter in an osteoblast cell line. Simvastatin, mavastatin, fluvastatin, and lovastatin all have been shown to stimulate bone formation.^(15,16) Also, the proangiogenic effect of statins may increase bone formation as they increase proliferation and differentiation of progenitors of endothelial cells.⁽¹⁷⁾ Moreover simvastatin helps in maintaining intact actin cytoskeletons and enhancing cell rigidity which are crucial in simvastatin-induced osteogenesis.⁽¹⁸⁾ **Ayukawa et al.** reported that systemic administration of simvastatin successfully activated osteogenesis around the titanium implant in rat models. However, systemic administration of simvastatin required much higher concentration for osteogenesis than for hyperlipidemia, so its topical application could be more effective rather than traditional oral doses.⁽¹⁹⁻²²⁾ Local application of simvastatin accelerated bone repair in parietal bone of rabbits and incisal sockets in rats.^(23 and 24)

Propolis is a substance made by the honeybee. It is composed of around 50% resins, 30% waxes, 10% essential oils, 5% pollen and 5% of other organic compounds. It has an antibacterial, antifungal, antiviral, antioxidant and anti-inflammatory properties.⁽²⁵⁻²⁷⁾ Systemic administration of propolis re-

duced periodontitis, alveolar bone loss and wound healing in rats.^(28 and 29) Topical application of alveolex (propolis 10%) associated with rhBMP-2 significantly increased bone repair in clavicle of rats.⁽³⁰⁾

Available data is documented regarding the potential effect of DDM on bone repair, while, no are available about the effects of DDM when used in combination with Statins or Propolis.^(8, 9) The aim of the present work was directed to study the effect of demineralized human dentin matrix graft alone and when combined with Statin or Propolis on bone repair.

MATERIALS AND METHODS

1- DDM preparation

DDM was obtained from human teeth without carious lesion or other pathology. The roots were cut and cleaned from dental pulp and periodontal ligament.⁽⁹⁾ Teeth roots were washed with saline at 2°C and then immersed in the 0.6N-hydrochloric acid solution at 2°C. Complete demineralization was done using EDTA and ensured by punching with needle through teeth (from 15-30 days). The specimens were then washed with distilled water for total acid removal and then cut into slices with frozen microtomy (Cryostat). These slices were immersed in a box filled with ethyl alcohol 70° ethyl alcohol and stored at 2°C until use within one month.^(4,5)

2- Statin

Statin solution was obtained by dissolving 10 mg simvastatin (Zocor® tablet, MERCK & CO, Inc, NJ, USA) in water for injection to the concentration of 2.5 mg/ml. The grafts were prepared 15 minutes before grafting by immersing DDM slices in statin solution.

3- Propolis

Propolis was obtained in liquid form (AL- Asal AL Barey CO, Kingdom Saudi Arabia). One drop was mixed with DDM graft and another one was

applied over the surface of the defect after graft insertion.

4- Animal Model

Thirty adult male rabbits (weight 2 to 2.5 kg) were used in this study. They were caged individually under optimum conditions in the animal house of the medical research center, Ain-Shams University. They were fed standard rabbit chows plus water ad-libitum. This experimental study was approved by Research Ethics Committee (REC), in Faculty of Dental Medicine for Girls.

5- Surgical Procedure

Intramuscular administration of anesthetic solution of ketamine (50 mg/kg) and Xylazin (20 mg/kg) was done. Then, skin of rabbits' legs was shaved and cleaned with a mixture of iodine and 70% ethanol. The upper part of the medial surface of rabbits' tibias were exposed by making a 5 cm linear incision through the skin, fascia and periosteum . Sterile round burs no. 4 were used to create two holes in each tibia, under sufficient coolant, by means of turbine powered hand piece. Four holes were drilled in each rabbit; one was left empty and used as control, while the DDM slices were packed in the other. Third hole was packed with DDM slices saturated with statin and the fourth was packed with DDM mixed with Propolis.

6- Grouping

Ten animals were sacrificed at 2, 4 and 6 weeks postoperatively. Experimental bone segments were cut out and put in labeled containers named; control, DDM, DDM/statin and DDM/propolis groups. Half of the specimens (5 rabbits) were fixed in 10% calcium formol, decalcified in EDTA and processed for H&E stain for histological and morphometric analysis. The other five rabbits from all groups were fixed in 2.5% glutraldehyde in phosphate buffer (PH7.4), dehydrated then gold sputter-coated to be examined by SEM.

7- Histomorphometric Analysis

Histological examination of the area of newly formed bone in the region of bone repair for each specimen. Then, the area of new bone formation and bone trabeculae were examined using objective lense of magnification 10x (total magnification of 100). Five fields were measured from each specimen and the area percentage was calculated. Image analysis was done using Leica Qwin 500 image analyzer computer system (England) at department of Oral Pathology, Faculty of Dental Medicine Ain-Shams University. The histomorphometric results were analyzed using ANOVA and Tukey test. The level of significance used was P < .05.

8- Scanning Electron Microscopy:

Bone specimens of five experimental rabbits from all groups were examined by SEM Philips XL 30 at the Central Laboratories for Research and Mining, Ministry of petroleum.

Histomorphometric Results:

The histomorphometric analysis measured mean bone area percent in the area of newly formed bone tissue. Analysis of the results (Fig.1) demonstrated that, DDM/Statin group presented the greatest mean bone area percent compared to other groups at each of the 3 time points. At 2 weeks, the least mean bone

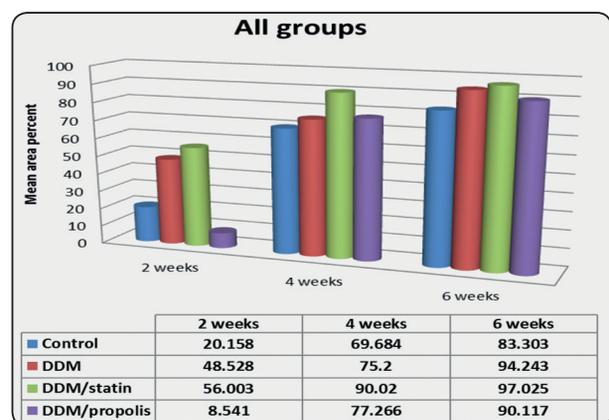


Fig. (1) Column chart showing mean bone area percent in different groups at the 3time intervals.

area percent was recorded in the DDM/Propolis group. Tukey's post hoc test revealed a statistically significant difference between each 2 holes. At 4 weeks, the lowest mean was recorded in control and there was no significant difference between mean bone areas in DDM and DDM/propolis holes. At 6 weeks, the lowest mean was recorded in control holes. There was no significant difference in the mean bone area between DDM and DDM/Statin, nor between DDM and DDM/Propolis.

Scanning Electron Microscopic results

After 2 weeks, control group showed thin bony cap covering the defective area with multiple empty spaces. The interface between old and new bones was clearly demarcated by longitudinal clefts. The surgical holes containing DDM, DDM/Statin and DDM/Propolis appeared to be closed with a meshwork of collagen fibers and new bone trabeculae. The surgical holes grafted by DDM and Propolis appeared in a lower level than the old bone. DDM slices could be seen at high magnification

within the voids in all DDM groups with clear presence of mineral crystals in DDM/Statin groups and reversal lines indicating active bone remodeling were seen (Figs. 2-4). At 4 weeks, both control and DDM/Propolis surgical holes appeared closed with new bone showing rough uneven texture with multiple irregularly outlined spaces (Figs.5-7). The DDM bone defects were closed with new bone with relatively smooth surface and haversian canals and collagen fibers. The surface in DDM/Statin surgical holes appeared closed with well oriented new bone. At 6 weeks, in the control group, the new bone surface appeared elevated, rough with multiple irregular spaces or clefts and wide haversian canals. On the hand, the DDM and DDM/Statin grafted holes presented well organized bone surface continuous with the surface of the adjacent old bone with few spaces observed in the DDM samples. The surface of the new bone covering the DDM and Propolis surgical holes appeared disorganized, enclosing some spaces and partially covered by thick collagen.

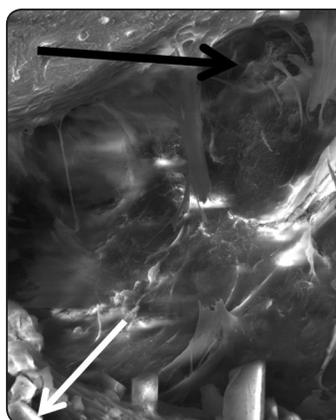


Fig. (2) A SEM at void of DDM/Statin hole at 2 weeks showing: DDM slice (black arrows) and mineral crystals (white arrow). (Orig. Mag. X1000).

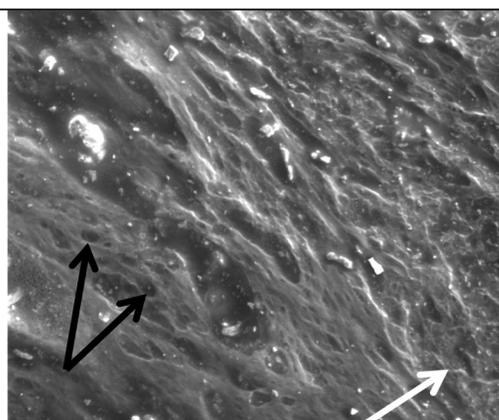


Fig. (3) A SEM of DDM/Statin group at 4 weeks showing: well oriented bone trabeculae enclosing haversian canals (black arrows) and reversal line (white arrow). (Orig. Mag. X250).

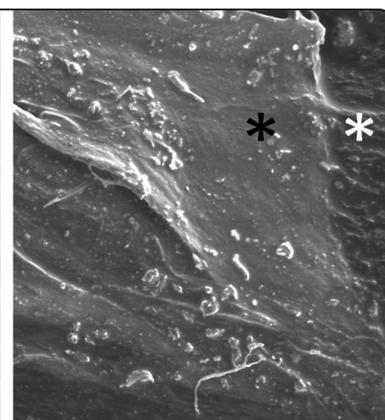


Fig. (4) A SEM of DDM/Statin at 6 weeks showing: well-organized new bone surface (black asterisk) running with the old bone (white asterisk). (Orig. Mag. X250).

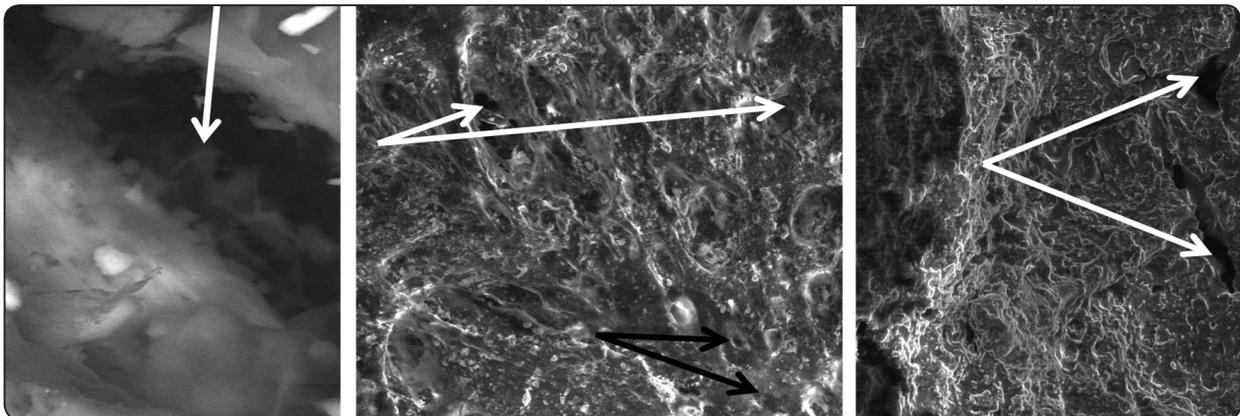


Fig. (5) A SEM at void of DDM/Propolis hole at 2 weeks showing: DDM slice (arrow) (Orig. Mag. X2000)

Fig. (6) A SEM of DDM/Propolis group at 4 weeks showing: disorganized bone trabeculae enclosing haversian canals (black arrows) and multiple spaces (white arrow). (Orig. Mag. X250).

Fig. (7) A SEM of DDM/Propolis at 6 weeks showing: disorganized new bone surface with some spaces (arrows). (Orig. Mag. X250).

DISCUSSION

Demineralization of dentin matrix exposed its organic matrix components like bone morphogenic proteins, noncollagenous proteins such as osteocalcin and osteonectin, and other growth factors. These factors induce bone formation and calcification.^(1,6,9) DDM was used as xenograft with ideal biocompatibility and negligible host immune reaction. Dentin collagen is the hardest among the body collagens as the dentin collagen did not expand in 0.6N HCl solution, while the expansion rates of skin collagen and bone collagen were about 4 and 1.2 times, respectively. The presence of the cell-adhesion domain sequence arginine glycine aspartic acid preferred by mesenchymal cells as anchorage-surface in addition to the hardness of dentin collagen provided suitable surface for bone matrix deposition.⁽¹²⁾

Rabbits were good model for the study, as they were easily available, housed and handled. Tibia represented an ideal site for the experiment as it was protected from contamination or trauma as in case of jaws.

The purpose of the histomorphometric analysis was to measure the quantity of newly formed bone

matrix in the bone defects and provide necessary data for statistical analysis. Histomorphometric results showed that after 2 weeks, DDM/Statin bone graft has greatest mean bone area percent followed by DDM, control and DDM/Propolis respectively. This was in agreement with Gomes et al results as there was statistically significant increase in mean bone area percentage in defects grafted with DDM alone compared to control at 15, 30 and 90 day.⁽⁵⁾ After 4 weeks, DDM/Statin reported the highest percent of mean bone area. However, DDM/Propolis group after 4 weeks recorded a significant increase in mean bone area when compared to the same group after 2 weeks, the percentage increased from 8.5% to 77.2%. After 6 weeks, DDM/statin group was still of highest percent of mean bone area. No significant difference in the mean bone area recorded between DDM/Statin and DDM nor DDM/Propolis. Moreover, this histomorphometric study did not express the quality of bone formed as the bone density was not measured.

The scanning electron microscopic results carried out in the current study provided good support for histomorphometric results however; they were only concerned with the surface topography. Examination of the demineralized dentin surface

using (SEM) revealed exposed dentinal tubules and the fiber bundles of both inter and peritubular dentin became loose, thus providing channels for releasing proteins and growth factors.⁽¹¹⁾

At 2 weeks DDM group showed pronounced new bone formation through the whole and also bone was formed on the periosteal and the endosteal surfaces of the adjacent old pre-existing bone which might be the result of stimulation of the osteogenic cells in the periosteum and endosteum. This enhancement of bone repair was in accordance with several previous studies.^(2,9,10) Gomes et al. observed an increase in the osteogenic cell population after DDM implantation.⁽⁵⁾ Researchers believed that the use of DDM as a graft induces neovascularization and differentiation of undifferentiated mesenchymal cells in the perivascular region of the newly formed vessels into osteoblasts.^(1,4,5,7)

When DDM graft was combined with statin, bone formation became more evident than DDM alone. However, this osteogenic activity of Statin was reported before by several studies.^(15, 19,22-24) On the other hand, Von Stechow et al. reported that systemic simvastatin failed to stimulate bone formation in ovariectomized mice.⁽²¹⁾ Statins exert their bone anabolic effects by differentiating mesenchymal cells into osteoblasts via up-regulating bone morphogenic protein-2 and protecting osteoblasts from apoptosis. In addition, Statins have anti-osteoclastic by reducing the osteoclast differentiation and activity.^(16,20) Statins also increase proliferation and differentiation of progenitors of endothelial cells providing new vessels required for new bone formation.⁽¹⁷⁾ Recently, the synergistic effect of simvastatin was attributed to activation of RhoA signaling that increases the cytoskeletal tension, which plays a crucial role in the osteogenic differentiation of mesenchymal stem cells.⁽¹⁸⁾

Addition of Propolis to the DDM graft in the same group caused marked delay in bone formation throughout the defective area, where the DDM slices

appeared surrounded by dense fibrous tissue. Bone matrix deposition on the surface of DDM slices was seen only in the depth of the defect and adjacent to the old bone (near to the bone marrow or adjacent vessels of old bone as a source of blood supply). Magro-Filho and Carvalho reported that Propolis application has no effect on socket wound healing.⁽²⁹⁾ Pereira et al. found that topical application of Propolis alone to bone defects in rats did not improve bone repair.⁽²⁶⁾ Furthermore, Propolis showed strong suppressive effects against vasoendothelial growth factor which induces angiogenesis.⁽³¹⁾ This may be responsible for the delay in bone repair in defective area away from bone marrow or blood supply.

Scanning electron microscopic results at 4 weeks of all groups showed better healing at the periphery more than the center. The surface of the new bone showed marked variation between different groups as in the histological analysis. The best surface texture was seen in DDM/Statin surgical holes which appeared closed with well oriented new bone. Areas of bone remodeling were represented by bright reversal lines. Cement or reversal line is deposited on the scalloped surface of the old bone, created by osteoclastic resorption, to anchor newly deposited bone onto the old bone surface. Previous studies described it to be less mineralized than the surrounding bone and its functions were energy absorption, viscous damping and making the bone more flexible by allowing movement between osteons.^(32,33) Based on recent studies, reversal lines are considered more mineralized compared to the surrounding collagenous bone allowing the formation of microcracks on the cement line instead of in osteons and/or interstitial lamellae to arrest the propagation of microcracks during fatigue.⁽³⁴⁻³⁶⁾ This brightness of reversal lines in the present study may be due to hypermineralized or collagen deficient areas with respect to surrounding bone.⁽³⁴⁾

After 6 weeks, Scanning electron microscopic examination revealed multiple rough irregular bony spaces in both control and DDM/Propolis groups. Collagen fibers appeared covering the surface

in many areas in DDM/Propolis group. In DDM group, the surgical holes were totally filled with new organized architecture bone and only few spaces were seen. In DDM/Statin group, best results were obtained and represented by new homogenous well organized bone surface, continuous with old bone.

CONCLUSION

Positive effect of DDM on bone repair was obtained. The combination of statin with DDM resulted in synergistic effect that greatly promoted bone healing. On the other hand, topical application of Propolis with DDM delay bone repair in early stages.

RECOMMENDATIONS

Further investigations are recommended to investigate biological response of autogenous demineralized dentin matrix, study quality and density of formed bone and determine the effective topical statin dose.

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