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The Effect of Human Demineralized Dentin Matrix Scaffold Alone and Combined with Concentrated Growth Factor on Bone Repair in Rabbits

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ABSTRACT

Purpose: This study aimed to evaluate the potential effect of Human Demineralized Dentin Scaffold (HDDS) alone and when combined with Concentrated Growth Factor (CGF) on repair of bony defects in the tibia of rabbits, histologically, by using scanning electron microscope and computed tomography. **Materials and Methods:** Ten adult male rabbits (weight 2 to 2.5 kg) were used in this study. Ethics committee approval of Faculty of Dental Medicine For Girls as obtained with code number (REC-BI-19-01). In each rabbit two holes were created (one in each tibia). In five rabbits, the first control hole was left empty and the second was packed with CGF. In the other five rabbits, one hole was packed with DDM slices from anonymous human permanent teeth without carious lesion or other pathology and the second was packed with DDM and CGF. The rabbits were euthanized at 1 and 6 weeks postoperatively and the bone samples were processed for histological analysis. **Results:** Faster bone repair was occurred in the experimental groups. In all groups, Osteoid tissue formation was occurred at one week, and osseous tissue formation was seen filling the bone defects at 6 weeks. The best results after 6 weeks appeared in surgical bone defects grafted with HDDS and CGF where the new bone showed better organization than the HDDS alone and CGF alone and most of the formed osteons were fully compacted. **Conclusion:** Combining Human Demineralized Dentin Scaffold (HDDS) with concentrated growth factor (CGF) improved graft biomaterial when used as xenograft and it became more effective through inducing a faster bone repair.

KEYWORDS

Human Demineralized Dentin Scaffold, Concentrated growth factor, Bone repair

- Paper extracted from Doctor thesis titled. The Effect of Human Demineralized Dentin Matrix Scaffold alone and Combined with Concentrated Growth Factor on Bone Repair in Rabbits

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INTRODUCTION

Bone is a complex tissue that has a special composition. It is composed of an organic collagen phase and inorganic compounds. There are many functions of bone skeleton in the human body, it is necessary for movement and for the protection of internal organs [1].

Bone defects may result due to several etiological factors as trauma, diseases, tumor resection or fractures, it is important to regenerate damaged bones. Natural human bone grafts are valuable sources for regeneration, but there is always a limit of bones that can be formed, the surgical steps are complicated, and recovery can be difficult. There is also probability for disease transfer. Therefore, the use of bone substitutes represents a golden solution to overcome these problems [2,3].

One of the ideal bone substitutes is Human Demineralized Dentin Scaffold (HDDS). It has been found to be an excellent biocompatible material with osteoinductive and osteoconductive potential. It has microporous structures named dentinal tubules that can be loaded with bone growth factors. It is acid insoluble scaffolds containing organic matrix [4-6].

Another bone substitute is Concentrated Growth Factor, one of the advanced products which contains several growth factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor, insulin-like growth factors, platelet-derived growth factor and chemokine receptor 4. Also, CGF devoid of the cell components of whole blood so no disease transfers through it. CGF represents a kind of fibrin plasma with the potential to fast tissue repair in many fields, such as bone regeneration, implants, angiogenesis and has a role in dental pulp revascularization [7,8].

MATERIAL AND METHODS

Materials:

Human Demineralized Dentin Scaffold (HDDS) was obtained from anonymous human permanent teeth without carious lesion or other pathology. The roots were cut and cleaned from dental pulp and periodontal ligament. Permanent teeth roots were washed with saline at 2°C and then were immersed in the 0.6N-hydrochloric acid solution at 2°C until complete demineralization (from 15-30 days). The specimens were washed with distilled water for total acid removal. After this process, the HDDS was cut into slices with frozen microtomy (Model LTD; BRIGHT Cryostat; HUNTINCDON /ENGLAND) in the Faculty of Medicine in Al-Azhar University. These slices were immersed in a box filled with ethyl alcohol 70% and stored at 2°C until using it within one month [9].

Concentrated growth factor (CGF) was obtained from the heart blood of healthy male rabbit. Donor rabbit was anesthetized by intraperitoneal injection of 10% chloral hydrate (0.4 ml/100 g); blood was taken by cardiac puncture directly into a sterile tube without anticoagulants and immediately centrifuged in a special centrifuge device (MedifugeTM; Silfradent srl, Sofia, Italy) for approximately 13 minutes. The fibrin buffy coat phase of CGF was separated using scissors. The CGF glue was pressed between two glass slides, squeezing the liquid elements and obtaining the CGF membrane [10].

Animals

Ten adult male rabbits (weight 2 to 2.5 kg) were obtained from and housed in Modern Veterinary Office, Al Haram, Giza. Ethics committee approval of Faculty of Dental Medicine For Girls was obtained with code number (REC-BI-19-01). The rabbits were following the rules and regulations of the animal experimental studies including their facilities, diet and method of euthanization. The animals were caged individually in a specially designed wire cages and were fed standard rabbit chows (green vegetables and carrots) plus water libitum at room temperature and normal humidity. The animals were euthanized at 1 and 6 weeks (five rabbits in each time) (Fig. 1A).

Figure (1) A clinical picture of the rabbit tibial surgical defect

Surgical procedure:

First, intramuscular administration of anesthetic solution of ketamine (10 mg/kg) and xylazine. Second, surgical procedures were made. Before surgery, the skin was shaved and disinfected with iodine 7.5% solution. Then 4 cm linear incision was made through the skin, fascia and periosteum expose rabbit's tibia. Sterile round bur number 5 were used to create one hole in each tibia, under sufficient coolant, by means of turbine powered hand piece. The suturing of the skin was made in 2 layers, muscle and dermis after the grafting materials were grafted inside their holes. Immediately after the operation animals were given intramuscular injection of Analgin as an analgesic and Cefotaxime (10 mg/kg) once daily for 3 days as antibiotic. After these 3 days, the animals appeared normal and did not show any signs of discomfort[11].

Animals collection:

Animals of the experimental groups were euthanized by using anesthetic overdose of thiopental (40mg/kg). All tibias were dissected carefully and then the one hole in each tibia was separated.

Specimen preparation for histological analysis:

The bone specimens of experimental rabbits from all groups were fixed for 48 hours in 10% calcium formol solution, washed and put them in 10% EDTA for decalcification for 5 weeks. After decalcification, the specimens were dehydrated in Xylol and then embedded in Paraffin blocks. Paraffin sections (6 microns thickness) were cut and put on cleaned glass slides, then stained with (H&E) stain for descriptive histopathological evaluation of the total area of bone defect, under a light microscope.

RESULTS

Histological results

At one week:

- **Control group (GI):** In control group, irregular newly formed bone trabeculae were observed which had a distinct woven conformation. The trabeculae of newly formed bone at the central region were thinner than the trabeculae at the margin of the bone defect. The periosteum appeared to cover the newly formed bone trabeculae in some areas only. The new bone trabeculae appeared enclosing few wide marrow cavities containing bone marrow and RBCs. (Fig. 2A)
- **CGF group (GII):** While in this group newly formed woven bone trabeculae appeared more than the control group and distributed through the whole defect. Also, the periosteum appeared continuously to cover the newly formed bone trabeculae. A network structure of dense fibrin was observed here. Increased number of generated blood vessel as compared with control group. (Fig. 2B)
- **HDDS (GIII):** While in this group newly formed woven bone trabeculae appeared thicker than those observed in the control and CGF groups. HDDS slices were observed to be scattered through the defect. Megakaryocytes appeared in bone marrow**.** Sometimes dentinal tubules were evident in the HDDS slices. Furthermore, Bone matrix deposition was visible on the surface of the HDDS slices. (Fig. 2C)

- **HDDS and CGF group (GIV):** While in this group, the newly formed bone trabeculae were more apparent than in control group, CGF a lone and HDDS alone. The surgical bone defects appeared covered by network of collagen fibers below the irregular newly formed bone trabeculae and HDDS slices. Thick new bone trabeculae appeared deposited on the top of HDDS. Dentinal tubules of HDDS were clearly seen. Bone matrix appeared deposited on the surface of HDDS in the depth of the defect. (Fig. 2D)

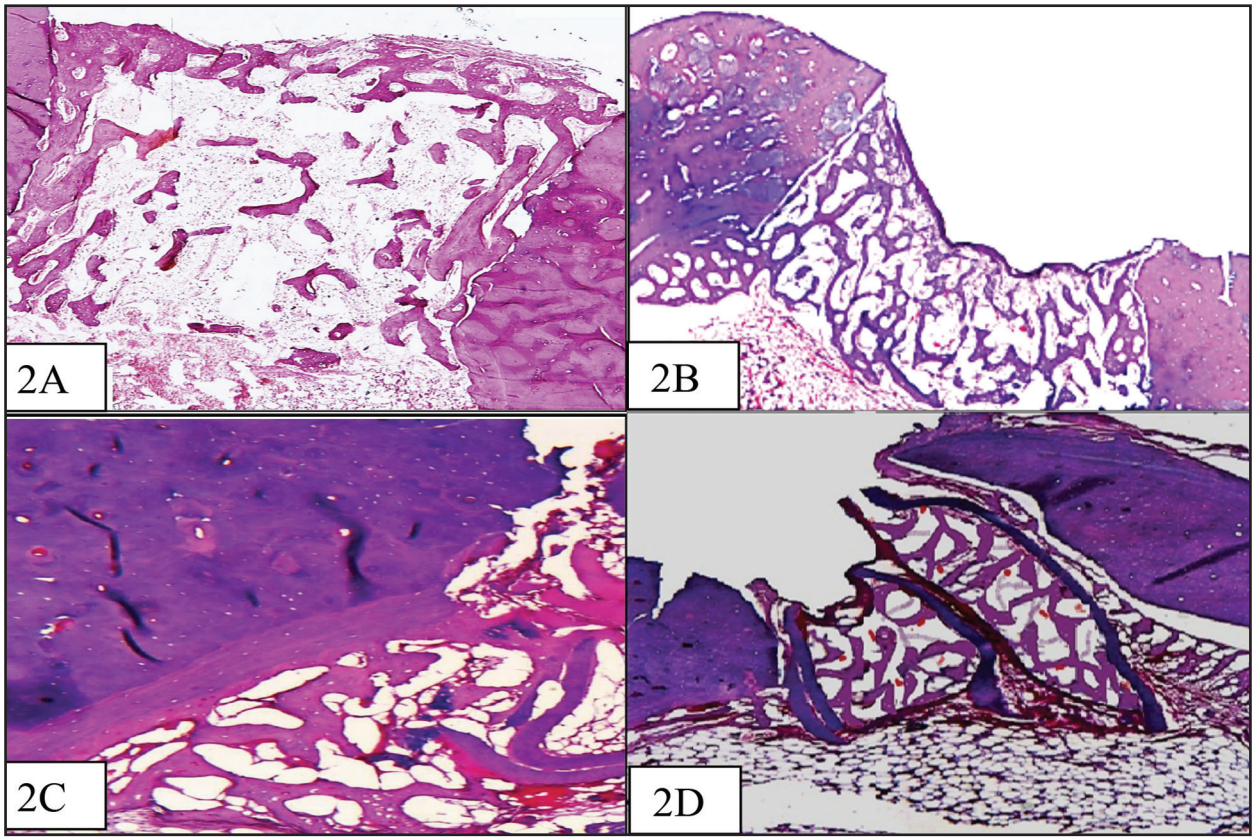

Figure (2): A photomicrograph of the rabbit tibial surgical defect: **[A]** Control group showing irregular newly formed bone trabeculae which had a woven conformation filled bone defect **[B]** CGF group showing the bone defect partial closed with new bone trabeculae which enclosing some marrow cavities. A porous network of fibrin was observed. Increased number of generated blood vessel as compared with control group. **[C]** HDDS group showing more pronounced formation of new bone trabeculae compared to those observed in the control and CGF groups. HDDS slices were observed to be scattered through the defect. Primary (woven) bone trabeculae appeared not mineralized yet. **[D]** CGF and HDDS group showing, the newly formed bone trabeculae was more apparent than in control group, CGF and HDDS alone. The new bone trabeculae were thick The defect appeared covered by network of collagen fibers below the irregular newly formed bone trabeculae and HDDS slices. (H&E, Orig. Mag x40).

At six weeks:

- **Control group (GI):** The surgical defect of bone appeared totally filled with bone that was continuous with the old bone. Variable sized and shaped marrow spaces were visible through the whole bone thickness. Small islands of woven bone appeared. The bone which is formed didn't reach a mature form yet and appeared arranged in osteonal pattern in some areas. The marrow cavities appeared either empty or involving fibrocellular marrow. (Fig. 3A)
- **CGF group (GII):** While in this group, the formed bone appeared in mature lamellar pattern with clearly recognized haversian system conformation and lined with endosteum as compared with control group. Haversian

canals surrounded by osteocytes with normal architecture.The surface appeared regular and covered by thick layer of periosteum. Resting lines and reversal line could be seen**.** Few number of generated blood vessel appeared in upper part of the defect. (Fig. 3B)

- **HDDS group (GIII):** While in this group, the defect appeared filled by new bone with clear haversian systems. Fibrocellular marrow and wide marrow cavities were mainly observed in the new bone but less than control group and more than CGF group. The new bone surface appeared relatively regular Difference in stainability was present not only between old and new bones, but also within the newly formed bone trabeculae. Typical osteons formed of central haversian canals surrounded by concentric bone lamellae and reversal line were seen. (Fig. 3C)
- **HDDS and CGF group (GIV):** While in this group, the surgical bone defects appeared totally filled by compact bone without marrow cavities as seen in the previous groups. The bone appeared in mature lamellar pattern with clearly recognized haversian system conformation and lined with endosteum. Haversian canals surrounded by osteocytes with normal architecture. The surface appeared regular and covered by thin layer of periosteum. Resting lines and reversal line could be seen. (Fig. 3D)

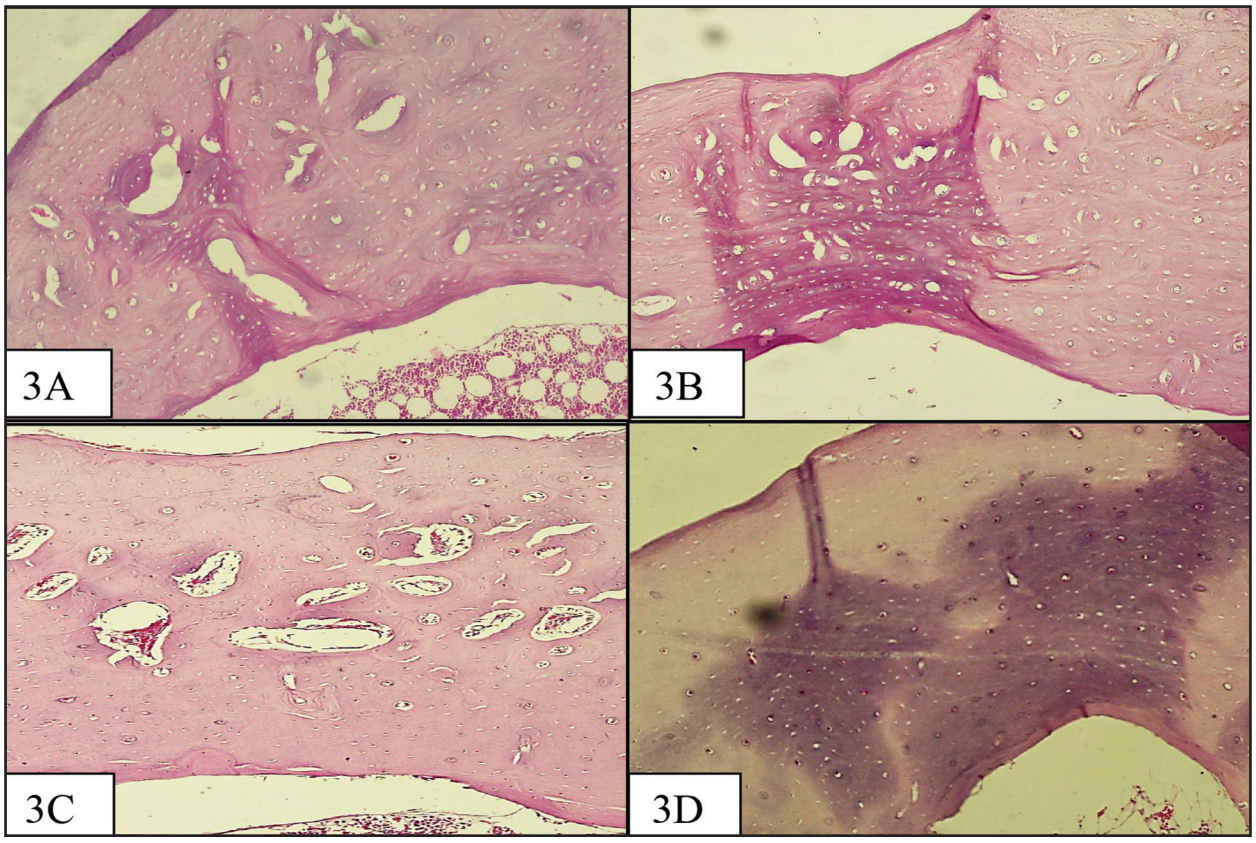

Figure (3): A photomicrograph of the rabbit tibial surgical defect: **[A]** Control group showing the defect appeared totally filled with bone. Variable sized and shaped marrow spaces were visible. The newly formed bone did not reach the mature form yet and appeared arranged in osteonal pattern in some areas **[B]** CGF group showing the surgical bone defects appeared totally filled by bone. The bone appeared in mature lamellar pattern with clearly recognized haversian system conformation**.** Few numbers of generated blood vessel appeared **[C]** HDDS group showing the surgical bone defects appeared filled by new bone with clear haversian systems. Fibrocellular marrow and wide marrow cavities were mainly observed. Typical osteons formed of central haversian canals surrounded by concentric bone lamellae and reversal line were seen. **[D]** CGF and HDDS group showing the defects appeared totally filled by bone. The bone appeared in mature lamellar pattern with clearly recognized haversian system conformation. Haversian canals surrounded by osteocytes with normal architecture and multiple resting lines were clear. (H&E, Orig. Mag x150).

DISCUSSION

Tissue regeneration involve three important elements. These elements are a scaffold, stem cells which contained in the scaffold and growth factors to act on these cells. Various materials have been used for regeneration of bone, like autogenous bone grafts and allograft bone. However, these techniques for bone grafting provide unsatisfactory results so the need for more better biomaterial increased, HDDS has been used as it was reported to contain growth factors [12].

In the present study, HDDS was used as xenograft in bone defects of rabbits and showed proper biocompatibility appeared in that HDDS graft formed new bone without host immune reaction. This was in accordance with several previous studies which used HDDS as xenograft for damaging bone with increase success rate. Human Demineralized Dentin Scaffold has advantages such as osteoinduction, safe material, no risk of infection transfer, easy grafted in the surgical site [13].

Demineralized Dentin Scaffold induces undifferentiated cells to differentiate into bone forming cells and then into cartilage and bone. The induction process by demineralized dentin is better than mineralized dentin. Moreover, the demineralization decreases the inorganic part which allow osteoblast adhesion to form bone and the resorption of dentin. Some investigators introduced several methods for dentin demineralization, the most favorable osteoinduction of them is 0.6 N HCl [14].

Some authors believe that the use of slices of HDDS as a graft induces a neovascularization inside the bone defect and that undifferentiated mesenchymal cells in the perivascular region of the newly formed vessels could be induced to differentiate into osteoblasts by the action of growth factors such as BMP from the HDDS [15].

The third generation of platelet is CGF which has more growth factor than the second-generation platelet (PRF) and the first-generation platelet (PRP). Concentrated Growth Factor can be considered as an ingenious biomaterial for tissue regeneration. The combination of DDM and CGF in our study improved bone regeneration [16]. The advantages of CGF are easily prepared, autologous and low cost [17].

The three major elements to regenerate bone are using synthetic bone graft substitutes (BGS), combining bioactive molecules with a carrier that is mostly an extracellular matrix protein and combining stem cells with a carrier. Each of these approaches is more important for the healing of bone defects, which is provided either by bioactive molecules, stem cells, or a combination of both. The combination and application of osteoinductive, osteoconductive and osteogenic elements are necessary for tissue engineering as in this study which investigate application of CGF on HDDS. Natural scaffolds such as HDDS contain collagenous protein and growth factors and application of concentrated growth factor which contain stem cell, can differentiate into osteogenic cell [18].

Rabbit is used as animal model in this study, and it is used as a first choice for musculoskeletal research. The bone mineral density is similar between rabbits and human. Rabbit has faster skeletal change and bone repair in comparison with other animals. Rabbits are available in many areas so rabbits the first choice for investigator to do in vivo experiment [19].

The tibia was chosen instead of jaw due to its similarity to human tibia, it provides a beneficial surgical site, since it is not affected by bacterial infection and trauma from chewing, also the double layer suturing (first deep soft tissue and then the skin) prevented unwanted site exposure. In this study, the control defects were left empty to be occupied by the blood clot without insertion of any material because in a previous study, when an inert material as Carbopol was inserted into sockets instead of normal blood clot, it did not make any changes in the amount of newly formed bone trabeculae[20] .

Osteoid tissue formation was seen in the bone defect at one week of experimental groups, and osseous tissue formation was seen filled the bone defect at six weeks. Also, bone formation occurred adjacent to HDDS. Bone repair occurred quicker in the experimental groups than the control. According to some researchers, BMP remain active for 10 years if stored at room temperature so teeth that are extracted at a young age for any cause is used to prepare HDDS then HDDS can be stored frozen by using liquid nitrogen for long periods and later thawed to be used as a graft material [21].

Compared with control group, the mature lamellar bone was increased and the graft material (HDDS) was decreased. HDDS has been used as a bone graft rather than autogenous bone and available in particulate and block form. Combining HDDS and CGF showed the fastest bone repair and the formed compact lamellar bone without space [22]

CONCLUSION

Demineralized Dentin Scaffold is proved to be an effective graft biomaterial when used as xenograft. The third-generation platelet (CGF) has a higher GF concentration than the first-generation platelet (PRP) and the second-generation platelet (PRF). Concentrated growth factor (CGF) has dense three-dimensional network of the fibrin which improves neovascularization. Angiogenic capacity is promoted by neovascularization and accelerate bone repair. Demineralized Dentin Scaffold is a bone substitute that be used as a scaffold carring concentrated growth factor by inducing new bone repair.

DECLARATION:

No conflict of interest.

No fund was received for this study.

RECOMMENDATION

Further investigation tools are recommended for the following: to widen the range of animals subjected to research for their biological response to DDM scaffold and better results are expected if it is used as autogenous one and to study the quality of the formed bone. to determine the behavior of CGF when used in the repair of bone defect in humans.

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