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Role of Coral, Demineralized Calf Fetal Growth Plate, and a Combination of the Two in Healing of Bone Defects in Rabbits

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Abstract

Background: Currently, coral is used as an osteoconductive material. Demineralized calf fetal growth plate (DCFGP) has also been used as a source of osteoinduction.
Objectives: This study examined the effect of a combination of DCFGP and coral in order to evaluate the associated bone healing properties in a critical size bone defect model in rabbits.
Methods: A critical size defect of up to 10 mm in length was created in the middle portion of the radial bone of 15 rabbits. The aforementioned defects were then filled with either coral, coral-DCFGP, or DCFGP alone. Radiographs of each forelimb were taken postoperatively every two weeks until the 8th week post-injury in order to evaluate the radiographic criteria for bone healing in the defected area. All rabbits were euthanized during the 8th postoperative week, and the treated radial bones were harvested for gross and histopathological evaluation.
Results: Macroscopic and histopathological evaluation did not reveal any significant differences between the three groups. The radiological evaluation of bone formation and the remodeling criteria revealed that there was a significant difference at the 6th and 8th postoperative weeks ($P < 0.05$). The coral-DCFGP group was superior to the other groups at the 6th and 8th postoperative weeks.
Conclusions: The radiological findings of the present study showed that the use of a coral-DCFGP mixture led to superior bone defect healing and rapid graft incorporation when compared to the use of solely coral or DCFGP.

Keywords: Coral, DCFGP, Radius, Bone Healing, Rabbit

1. Background

An ideal bone grafting material has osteoinductive as well as osteoconductive properties. Such a material should provide mechanical scaffold for vascularization and tissue infiltration, and also serve as a carrier for releasing essential growth factors (1, 2). Currently, there are several biomaterials available for enhancing bone healing, including collagen base materials, demineralized bone matrix, platelet gel, titanium, polymer-based materials, nanohydroxyapatite, demineralized bovine bone matrix, and ceramic-based material (3-7). Each of these biomaterials has advantages and disadvantages. For example, ceramic and polymer-based materials have more osteoconductive and less osteoinductive properties, and they also have unexpected degradation rates accompanied by less mechanical properties. Additionally, other problems for protein- or growth factor-based materials include the need for the concurrent use of an osteoconductive scaffold to support such materials (8, 9). Several studies have been conducted to assess bone regeneration. Some of these studies have involved the application of bone marrow in a static magnetic field (10), coral with human platelet rich plasma (11), hydroxyapatite with human platelet rich plasma (12), and omentum with adipose tissue stem cells.

There are some species of coral with a structure that resembles bone matrix structure. The calcium carbonate skeletons of fourteen coral species out of over 2000 unique coral species have been studied as possible bone grafting substitutes. These species included Pocillopora, Acropora, Montipora, Porites, Goniopora, Fungia, Polyphyllia, Favites, Acanthastrea, Lobophyllia, and Turbinaria (13). In the Persian Gulf and Kish island, the most prominent coral species are *Porites lutea* and *P. compressa* (14, 15). The calcium carbonate skeleton ($CaCO_3$) of coral works as a hydroxyapatite material. For example, it is biocompatible and osteoconductive, but it has no osteoinductive proper-

ties (16). Previous animal studies have shown that the coral resorption rate differs from that of hydroxyapatite (17).

In a recent study, we showed that the hydroxyapatite-DCFGP group was superior to the other groups tested at the 8th postoperative week, in terms of the radiological criteria (18).

To the best of our knowledge, no previous study has evaluated the concurrent use of calcium carbonate and DCFGP for bone defect healing.

2. Objectives

The present study was designed to evaluate the bone healing properties of DCFGP in combination with coral. The coral has some beneficial properties such as porous architecture, good biomechanical support, absorbability, and good biocompatible; therefore, it was selected as the scaffold. We hypothesized that a combination of coral as an osteoconductive material and DCFGP as an osteoinductive material could lead to better bone formation in the bone defect.

3. Methods

In the present study, fifteen white New Zealand (NZW) rabbits (18 months old, mixed sex, weighing 2.5 $\pm$ 0.5 kg) were used. They were fed with pellets during the study. All of the animals were randomly divided into three groups: DCFGP group (n = 5), DCFGP-coral group (n = 5), and coral group (n = 5 group). The rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (40 mg/kg) and xylazine (5 mg/kg). After the aseptic preparation of the right forelimb, a craniomedial skin incision (5 cm incision) was made and the radial bone was exposed. An osteoperiosteal segmental defect of 10 mm in length was created on the diaphysis of each radius in order to create a critical size bone defect model (19). As the diameter of the radius of adult NZW rabbits is about 5 mm, a radial defect should be no less than 10 mm long for the creation of a critical size defect in a rabbit model (20). The defect was carefully checked and any residual periosteum was removed. Irrigation with saline then removed any residual materials. In the coral group, the bone defects were filled with a coral segment of the same length of the defect. In the coral-DCFGP group, the bone defects were filled with a coral segment and 0.5 mg of DCFGP powder, while the defects of the animals in the DCFGP group were filled with 1 mg of DCFGP powder. The ethical committee approved the design of the experiment and the treatment was line with established principles concerning "the care and use of animals."

3.1. Preparation of DCFGP

The demineralized calf fetal growth plate was prepared according to the methods used in previous studies by Bigham-Sadegh et al. (4, 18, 21, 22).

3.2. Coral Preparation

The coral exoskeleton (Porites sp.) was collected from the Persian Gulf, Kish island, Iran. The collected coral was fabricated into cone-shaped blocks of 5 mm in length and 4 mm in diameter. The fabricated corals were then autoclaved and stored in aluminum foil (23).

3.3. Postoperative Evaluation

3.3.1. Radiological Evaluation

For the evaluation of the radiological criteria for bone healing, we used a modified Lane and Sandhu scoring system (24) (Table 1). Radiographs were taken every two weeks following surgery until the 8th week post-injury.

Table 1. Modified Lane and Sandhu Radiological Scoring System

Bone Formation	
No evidence of bone formation	0
Bone formation occupying 25% of the defect	1
Bone formation occupying 50% of the defect	2
Bone formation occupying 75% of the defect	3
Bone formation occupying 100% of the defect	4
Union, proximal and distal union evaluated separately	
No union	0
Possible union	1
Radiographic union	2
Remodeling	
No evidence of remodeling	0
Remodeling of the medullary canal	1
Full remodeling of the cortex	2
Total points possible per category	
Bone formation	4
Proximal union	2
Distal union	2
Remodeling	2
Maximum Score	10

3.3.2. Macroscopical and Histopathological Evaluation

At eight weeks post-injury, all of the rabbits were euthanized and the treated bones were harvested for macroscopical and histopathological evaluation. Prior to

the histopathological evaluation, macroscopic scoring for gross signs of healing was performed by two examiners who were blinded to the experiments. The scoring system for the macroscopic evaluation of healing signs included: presence of bridging bone indicating a complete union (+3 score), presence of cartilage, soft tissue or cracks within the defect indicating a possible unstable union (+1 or +2 score), and complete instability at the defect site indicating no union (0 score).

After the macroscopical scoring, the specimens were referred for histopathological evaluation. The defected area was cut in a sagittal manner with a low speed electrical saw and then fixed in 10% neutral buffered formalin. A 15% buffered formic acid solution was used for the samples' decalcification. At the center of each specimen, two sections of 5 μm in thickness were cut and stained with hematoxylin and eosin. The stained samples were blindly examined and scored by two pathologists according to Emery's scoring system (25). The histopathological scoring criteria included: empty gap (score = 0), gap filled by fibrous connective tissue only (score = 1), more fibrous tissue than fibrocartilage (score = 2), more fibrocartilage than fibrous tissue (score = 3), fibrocartilage only (score = 4), more fibrocartilage than bone (score = 5), more bone than fibrocartilage (score = 6), and filled with only bone (score = 7).

4. Results

Ulna bone fracture was not observed at the radial bone defect site of any rabbits.

4.1. Radiological Findings

According to the radiological evaluations, there were no significant differences between the groups at the 2nd and 4th postoperative weeks. However, at the 6th and 8th postoperative weeks, the radiographs showed significant differences between the groups ($P < 0.05$) (Figure 1 and Table 2). The coral-DCFGP group was significantly ($P < 0.05$) superior to the coral and DCFGP groups. There were no significant differences between the DCFGP alone and coral alone groups at any postoperative day (Figure 1 and Table 2).

In the macroscopical evaluation, all of the rabbits showed different amounts of new bone formation and there were no statistically significant differences between the groups (Table 3).

While in the histopathologic evaluation the defects of the animals in the coral-DCFGP, coral, and DCFGP groups showed no significant differences in terms of the statistical analysis ($P > 0.05$) (Table 3), the coral-DCFGP group showed more cortical bone formation in comparison with the trabecular bone formation of the coral or DCFGP groups (Figure 2).

Overall, mature cortical bone was seen in all defects (Figure 2). In some of the rabbits, trabecular and woven bone with a histological union were seen in addition to cortical bone (Figure 2).

5. Discussion

In this study, the coral-DCFGP group showed greater radiologic healing of the rabbit radial bone defect in comparison to both the coral and DCFGP groups in the radiological evaluation. Our findings showed that the concurrent use of coral and DCFGP leads to favorable bone healing. We used a rabbit model for the critical defect of long bone healing in the present study. Rabbits are used as animal models in approximately 35% of all musculoskeletal studies (26). The radial, fibular, and calvarial bones of rabbits have been reported to be suitable because there is no need for external or internal fixation of the defect site after the experimental induction of the bone defect model (27-29).

We proposed that the concurrent use of DCFGP and coral in a critical size defect in the radial bone of rabbits could enhance bone formation. Recently, a study indicated that the submuscular implantation of demineralized bovine fetal growth plate led to ectopic bone formation without any complications (21). Also, in two previous studies segmental calf fetal growth plate was grafted into the radial bone defect and a positive bone healing process was observed by investigators (29, 30). A more recent study showed favorable bone defect healing with DCFGP in a rabbit model (22). In our study, the DCFGP group showed good bone healing similar to the other groups in the histopathological evaluation. Two previous studies also showed the presence of growth factor β (TGF- β) and bone morphogenetic proteins 2 and 7 in the human and rat fetal growth plates (31, 32). These three proteins can promote the chondroblastic differentiation of mesenchymal cells and so new bone could form via endochondral osteogenesis (33, 34).

Low molecular weight glycoproteins such as bone morphogenetic proteins (BMPs) were identified in the demineralized bone matrix (DBM) with osteoinductive properties. These proteins were cited in the mineral matrix of bone and, during the demineralization process, the proteins were exposed and can promote endochondral bone formation via accelerating the chondroblastic differentiation of mesenchymal cells (34, 35).

More recently, studies have shown the ectopic osteoinductive properties of calf fetal growth plate in a rat sub-

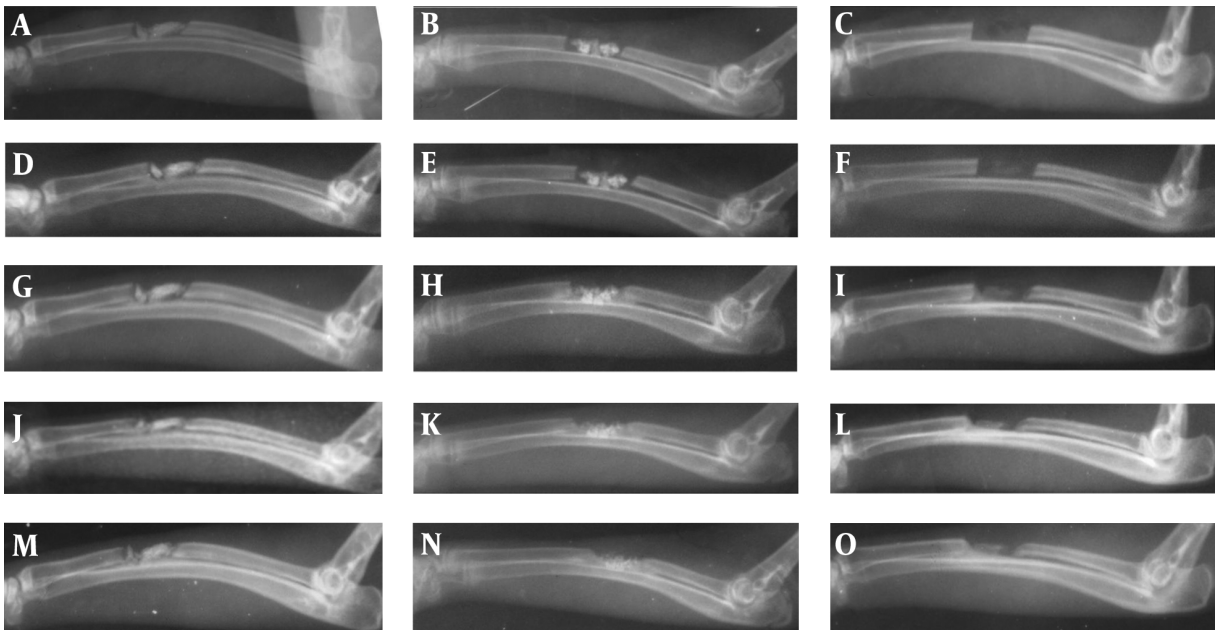

Figure 1. Radiographs taken of Group I (coral group), Group II (coral-DCFGP group), and Group III (DCFGP group): A-C, Groups I-III at day 0, respectively; D-F, Groups I-III at day 14, respectively; G-I, Groups I-III at day 28, respectively; J-L, Groups I-III at day 42, respectively; and M-O, Groups I-III at day 56, respectively.

Table 2. Radiological Findings Regarding the Healing of The Bone Defect (Sum of the Radiological Scores) at Various Post-Operative Intervals

	Med, Min - Max			P[a]
Postoperative weeks	**Coral Group, n = 5**	**Coral-DCFGP Group, n = 5**	**DCFGP Group, n = 5**	
2	2 (0 - 3)	3 (1 - 3)	1 (0 - 3)	0.2
4	5 (2 - 7)	8 (3 - 9)	4 (3 - 6)	0.1
6	5 (3 - 7)	8 (6 - 9)[b]	4 (3 - 6)	0.01[c]
8	6 (4 - 7)	10 (9 - 10)[d]	6 (4 - 7)	0.008[c]

[a] Kruskal-Wallis non-parametric ANOVA.
[b] Compared with the coral group (P = 0.02) and DCFGP group (P = 0.01) by the Mann-Whitney U test. The coral-DCFGP group was significantly (P < 0.05) superior to the hydroxyapatite and DCFGP alone groups.
[c] Significant P values.
[d] Compared with the coral group (P = 0.008) and DCFGP group (P = 0.008) by the Mann-Whitney U test. The coral-DCFGP group was significantly (P < 0.05) superior to the hydroxyapatite and DCFGP alone groups.

Table 3. Bone Measurements at the Macroscopic and Microscopic Levels

	Med, Min - Max			P
Bone type evaluation	**Coral Group, n = 5**	**Coral-DCFGP Group, n = 5**	**DCFGP Group, n = 5**	
Macroscopic union[a]	3 (2 - 3)	3 (3 - 3)	2 (2 - 3)	0.2
Microscopic evaluation[b]	5 (4 - 7)	6 (5 - 7)	4 (5 - 7)	0.1

[a] Complete union (+ 3 score), presence of cartilage, soft tissue or cracks within the defect indicating a possible unstable union (+ 1 or +2 score), complete instability at the defect site indicating nonunion (0 score).
[b] Empty (0 score), fibrous tissue only (1 score), more fibrous tissue than fibrocartilage (2 score), more fibrocartilage than fibrous tissue (3 score), fibrocartilage only (4 score), more fibrocartilage than bone (5 score), more bone than fibrocartilage (6 score), and bone only (7 score).

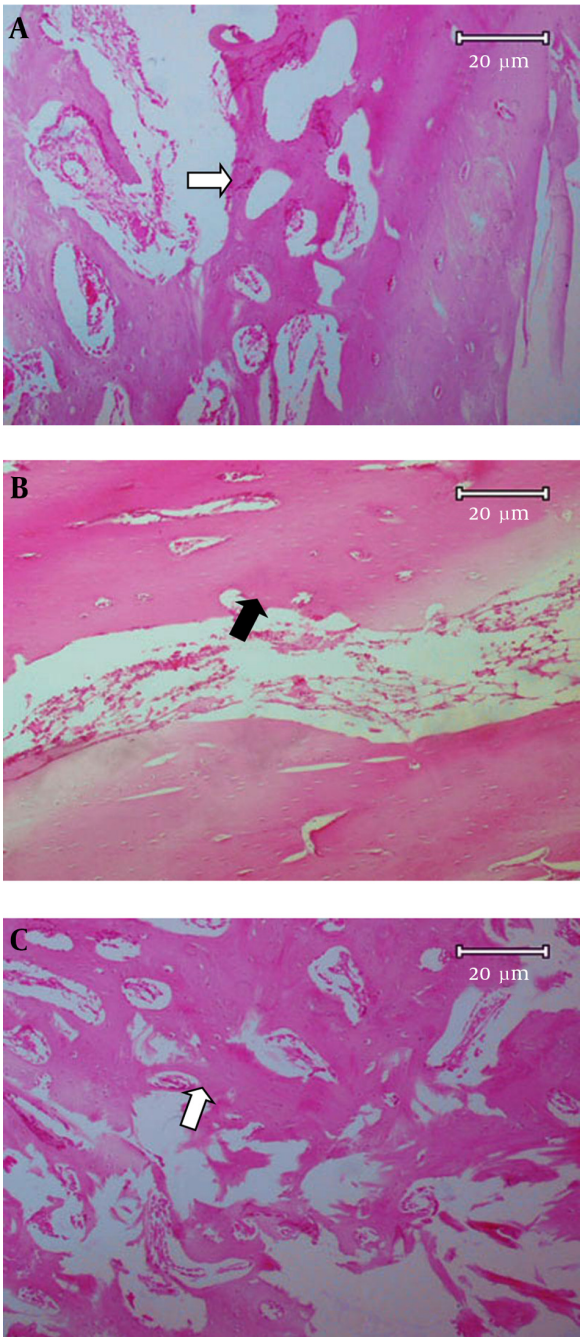

Figure 2. Micrographs of the treated bones after eight weeks. A, regenerated bone with the typical structure of the trabecular bone (note the white arrow) is seen in the defect from the coral group. B, cortical bone formation (note the black arrow) in the coral-DCFGP group, and C, trabecular bone (note the white arrow) in the DCFGP group (H and E staining) (10×).

muscular model (21) as well as enhanced bone healing in a rabbit bone defect model (22, 30). Two previous studies showed the presence of growth factor β (TGF-β) and bone morphogenetic proteins 2 and 7 in the human and rat fetal growth plates (31, 32). These three proteins promote the chondroblastic differentiation of mesenchymal cells and so new bone formation occurred via endochondral osteogenesis (33, 34). In our study, we supposed that the coral-DCFGP, calf fetal growth plate demineralization exposes the TGF-β and BMPs 2 and 7 into the injured site, which means it acts as an osteoinductive material while the coral acts as an osteoconductive material. Therefore, the bone healing process seen in the radiological evaluation of the coral-DCFGP group was superior to that seen in the other two groups. However, macroscopic and microscopic evaluation did not support this finding, and there were no significant differences between the groups at eight weeks post-injury. There are many studies demonstrating the successful use of coral for spinal fusion (36, 37), cranial surgery (38), and dentistry (39). However, there are also studies that showed coral to have only osteoconductive properties and found that the pores of coral were only filled with fibrous or fibrocartilage tissues and so bone formation had not occurred (40). Additionally, another previous study found that coral has only osteoconductive properties where it contacts with bone and so it is not sufficient for bone formation and union (40). Further, it has been shown that coral's calcium carbonate osteoconductive properties are the same as the osteoconductive properties of hydroxyapatite (41). Based on the findings of all these studies, we used coral as a scaffold for DCFGP.

In an ectopic bone formation model, it has been shown that by adding bone marrow to the coral, the osteogenesis properties of the coral were increased (41, 42). In our study, the addition of DCFGP to coral increased osteogenesis and favorable bone union was observed in the critical size bone defect of a rabbit model. Also, Parizi et al. (2012) showed that when platelet rich plasma was added to coral, it led to superior bone formation in comparison to the control groups (11). The results of these studies are similar to the findings of the present study.

In the present study, unexpected cortical bone and medullary canal formation was observed in some of the rabbits. Of course, good bone marrow stromal cell attachment, growth, and differentiation on the coral's surface has been previously shown in in-vitro studies (43). We suggested that adding DCFGP to coral induced the local stem cells to differentiate, and attach and grow into the coral pores and produce remodeled cortical bone.

Alper and colleagues showed suitable bone formation with the use of hydroxyapatite in combination with DBM after eight weeks of fracture healing in rats and rabbits

(44). Moore and colleagues showed that using autogenous cancellous bone in combination with hydroxyapatite led to superior bone formation in comparison with hydroxyapatite alone, and they further suggested that osteoconductive materials should be used in conjunction with osteoinductive materials (45). Another study found similar results (46). These studies support our radiological findings showing suitable bone defect healing. However, the macroscopical and histopathological evaluation did not reveal any significant differences between the three groups after eight weeks and actually showed favorable bone healing scores in all three groups.

The present study was limited by the fact that it lacked the evaluation of the healing bones via 3D micro CT scan, biomechanical properties, and immunohistochemistry. However, these useful tracking techniques could be applied in similar experiments in the future.

Although the macroscopical and histopathological evaluation did not reveal any significant differences between the three groups the radiological findings of the present study showed that the coral-DCFGP mixture led to superior bone defect healing and rapid graft incorporation in comparison to the use of solely coral or DCFGP.

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Footnote

Authors' Contribution: Study concept and design, all authors; acquisition of data, All authors; analysis and interpretation of data, Amin Bigham-Sadegh; drafting of the manuscript, Amin Bigham-Sadegh; critical revision of the manuscript for important intellectual content, Amin Bigham-Sadegh; statistical analysis, Ahmad-Reza Mohamadnia and Amin Bigham-Sadegh; administrative, technical, and material support, all authors; study supervision, Amin Bigham-Sadegh.

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