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RECONSTRUCTION OF BONE DEFECT WITH THE CORAL SCAFFOLD AND OSTEOBLASTS: AN EXPERIMENTAL STUDY IN RABBITS

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ABSTRACT
Demand for bone grafts to treat bone defects in cases of pathologically and injuries is increasing, but the source of bone graft derived from humans is not enough to supply the needs of patients. Therefore, research and create the equivalent of bone graft is a necessary work but also a long-term solution in the future. The present study was carried out with the goal of creating the equivalent bone graft using sea coral (*Porites lutea* species) as the substrate to contain osteoblasts derived from bone marrow to treat defects bones in the body of the rabbit femur bones. This is a controlled experimental study. Rabbits were divided into two groups, Group 1 (experimental rabbits), including the rabbit pieces were grafted by the coral contained osteoblasts. Group 2 (control group) consisting of rabbits were grafted by coral alone. Research results on the rabbits were evaluated at the time points of 1, 3 and 6 months by radiology and histology (H & E staining). From this study, we found that the group of rabbits that were grafted by coral containing autologous osteoblasts, the bone healing results occur faster and better quality of bone healing when compared with the control group at time study 1 month and 3 months. The study results will open up the prospects of clinical applications in the future for bone defects cases, this will be a long term solution and effective for patients when there is not enough bone graft source, in while autologous bone grafting is limited in terms of quantity.

KEYWORDS: sea coral, osteoblast, bone defect, grafting, reconstruction.

1. INTRODUCTION

Bone tissue is one kind of tissue in the human body that is capable of self-healing in the small bone defects. However, in the case of injury or illness caused the large bone defects, the bone tissue is not capable of self-repair without the use of interventions such as bone graft substitute.[1,2,11] However, bone grafts problems existing obstacles need to be overcome. If the use of autologous bone tissue, patients need bone tissue obtained from a different location on the body, which leads to prolonged surgery leads to the risk of bleeding, inflammation and slow wound recovery. Bone allografts are the next choice, but the type of bone tissue often fails to meet demand by depending on the number of donors and the risk of infectious diseases. Source grafts from animals is very rich but the risk of transmission of animal diseases are difficult to control.

Therefore, the application of various biomaterials for bone grafting is an essential demand. Besides, we also need to improve the quality of the bone graft substitution by adding cells to increase the efficiency of bone formation and help the bone healing occurs faster and more efficient.[1,2,4]

We conducted the study to evaluate the ability to regenerate bone tissue for bone defects by grafts created from the combination of sea coral (*Porites lutea* species) and autologous osteoblasts derived from bone marrow on the rabbit femur.[1] Since then, we have evaluated to be effective in clinical applications.

2. MATERIALS AND METHODS

2.1. Materials

Brown rabbits were collected from farms in Vietnam. Rabbit was chosen as the male rabbits, healthy, body weight from 2.0 to 2.5 kg, about 6 months old. Rabbits were housed and cared for in the same conditions.

Porites lutea corals were collected from Institute of Oceanography, they are manufactured in the bone graft substitution at The laboratory of Biomaterials, Pham Ngoc Thach University of Medicine.

2.2. Isolation of mesenchymal stem cells from the bone marrow of brown rabbit species

Rabbits were anesthetized by Zoletil 50 (VIRBAC Laboratories, France). Then, we used Betadine (Zuellig Pharma) to disinfect the area obtained in the knee bone

marrow. Using a syringe and needle 18G (Vihanmedico, HN, VN) to aspirate bone marrow. Acquisition of 0.5 ml of rabbit bone marrow. The steps had taken to ensure the requirements for aseptic manipulation.

Mononuclear cells were isolated from bone marrow by centrifugation with Ficoll-Hypaque solution (Amersham, Germany). After centrifugation, obtain the mononuclear cells fraction layer between Ficoll and plasma layers. Mononuclear cells were cultured in a cell culture medium consisting of DMEM / F12 (Gibco, USA), 10% FBS (Gibco, USA) and Pen / Strep (Sigma). Replace new cell culture medium every 3 days. After 7 days, cells were subcultured into new cell culture flaks.

2.3. The combination of Porites lutea coral and mesenchymal stem cells to create bone graft substitute

Cultured cells from the bone marrow subcultured to the second generation. These cells are harvested and transferred to the coral (0.5x0.5x0.5 cm in size) with a cell density of about $1x10^5$ cells/cm^3.

Bone grafts cultured in CO_2 incubation cabinet with cell culture medium consisting of DMEM/F12, 10% FBS and antibiotics for the first day. Then use the cell culture medium induced bone formation including DMEM / F12, 10% FBS, 10^{-8}M dexamethasone (Sigma), 10 mM/ml β-glycerol phosphate (Sigma), 50 ng/ml of ascorbic acid (Sigma), 10ng/ml FGF-9 (Sigma), 10^{-7}M vitamin D2 (MekomPharma, HCM City, VN) and antibiotics.[9] Fresh cell culture medium was replaced every 3 days.

2.4. The evaluation method of bone graft substitute

Histologic staining (H & E) to evaluate the distribution and development of cells inside the bone graft substitute; SEM to examine the adhesion, growth of cells on the surface of the bone graft substitute. In addition, we evaluated the presence of osteoblasts in bone graft substitution by determining the presence of the enzyme alkaline phosphatase via Fast Red Violet LB dye.

2.5. Transplantation of bone graft substitute in the body of the rabbit femur

Bone graft substitute cultured until day 21, it was used to implant in the body of the rabbit femur. Rabbits were divided into 2 groups: group 1 (group of rabbits implanted with bone graft substitute consisting of autologous osteoblasts and corals) and group 2 (only coral frame).

Both groups of rabbits this study will be taken care of housing, nutrition and care the same conditions. The rabbit studies followed at the time points 1, 3 and 6 months. At each time point, 3 rabbits from two research groups will be assessed by X-ray and biopsy tissue samples obtained for histology.

3. RESULTS

3.1. Findings isolated and cultured stem cells from the bone marrow of rabbit

After 1 day of cell culture, cell adhesion appears with an elongated shape, similar to the morphology of the fibroblasts. These cells are characterized by adhesion to the bottom of the flasks. After a week of culture, these cells can increase the number of cells by subculture into the new flask.

Based on the source acquisition (bone marrow) and cell morphology as well as the ability to attach to the bottom of the flasks, this is considered as mesenchymal stem cells.[5,7,8,12]

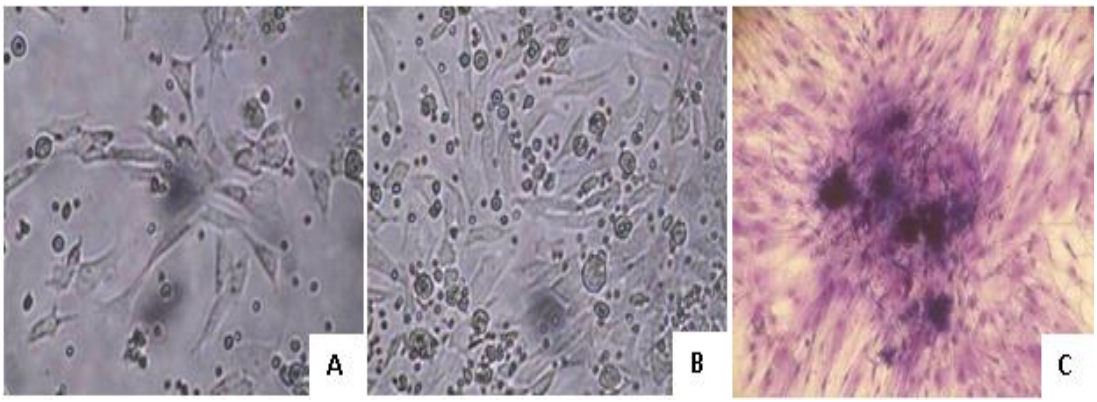

Figure 1: Results of culturing stem cells obtained from bone marrow of rabbit. (A). After 3 days cell culture (10X). (B). After a week of culture (10X). (C). Cells were stained with Giemsa to observe cell morphology (10X).

3.2. Results generated graft used to replace bone tissue

Based on histological results showed that the cells were cultured on coral growth and fairly evenly distributed. In addition, these cells also grow on the surface of the coral frame (by Giemsa staining). These cells were positive for Fast Red violet LB dyes, their cytoplasm was pink appearance characterized by the activity of the enzyme alkaline phosphatase (this is one of the markers used to identify the osteoblasts) (Figure 2).

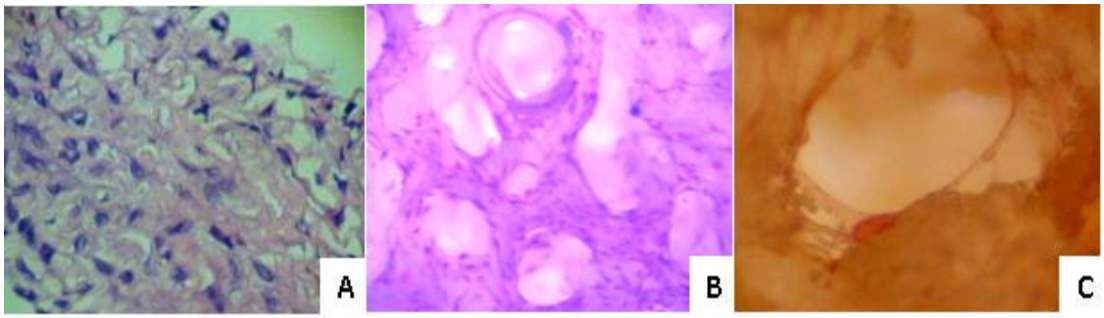

Figure 2. The results of the evaluation of bone graft substitution. (A). Cells were grown on scaffolds stained dark purple coral and distributed equally in coral frame (histological stain, 10X). (B). Dark purple staining cells, adhesion on surfaces and cracks of coral scaffolds (Giemsa staining, 4X). (C). Osteoblasts dyed dark pink by alkaline phosphatase activity of the enzyme reacts with Fast Red Violet LB dye (10X).

After 21 days of differentiation into osteoblasts directly on coral scaffolds, the growth of osteoblasts to form monolayers on the surface of the coral scaffolds (SEM) (Figure 3).

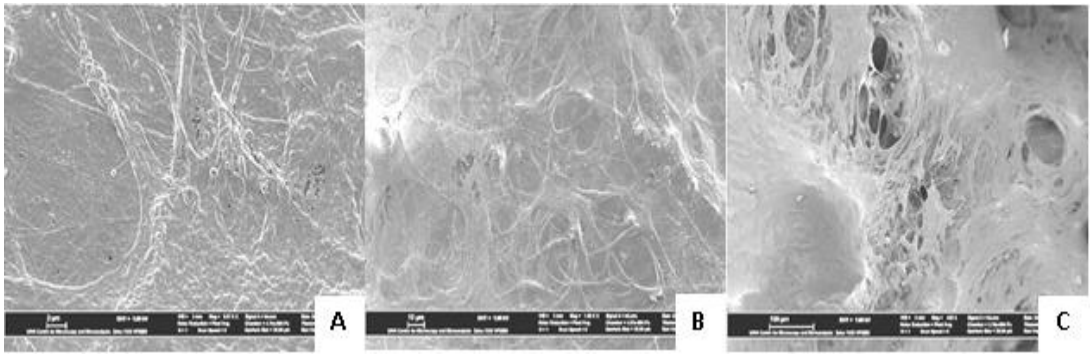

Figure: 3. SEM images of osteoblasts were cultured on the scaffold coral. (ASIAN). Cell adhesion and developed into monolayer on the surface of the coral frame (size 2 micron). (B). 10 micron size. (C). The single layer of cells on the surface and in the cavities of the coral frame (size 100 micron).

3.3. The assessment results in the rabbit model

Results were grafted bone graft in rabbits at 1 months

In the research group, results in X-ray film (Figure 4) showed that at both ends of the bone between the host bone and bone graft with good adhesion, bone graft is still seen quite clearly. The main difference between the two groups is the connection between bone graft and host tissue, which is very important to help stabilize graft, to the regeneration and repair of bone occurs efficiently.

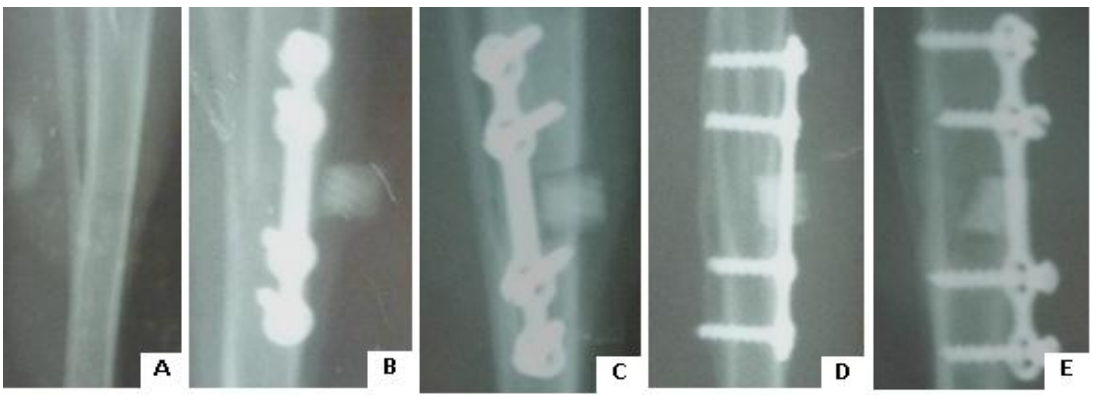

Figure 4. Results of transplants in rabbits at the time of a month. (A) The body of the rabbit femur before transplant surgery. (B) and (D). Bone grafts are created from autologous osteoblasts and coral. (C) and (E). Only coral samples.

Histologic results (Figure 5) showed that in the rabbit study group containing autologous osteoblasts, the bone graft structure remains stable. Bone graft appeared many new blood vessels within the graft. In addition, the emergence of more new bone formation with the bone cells (osteocytes) and the bone-forming cells (osteoblasts).

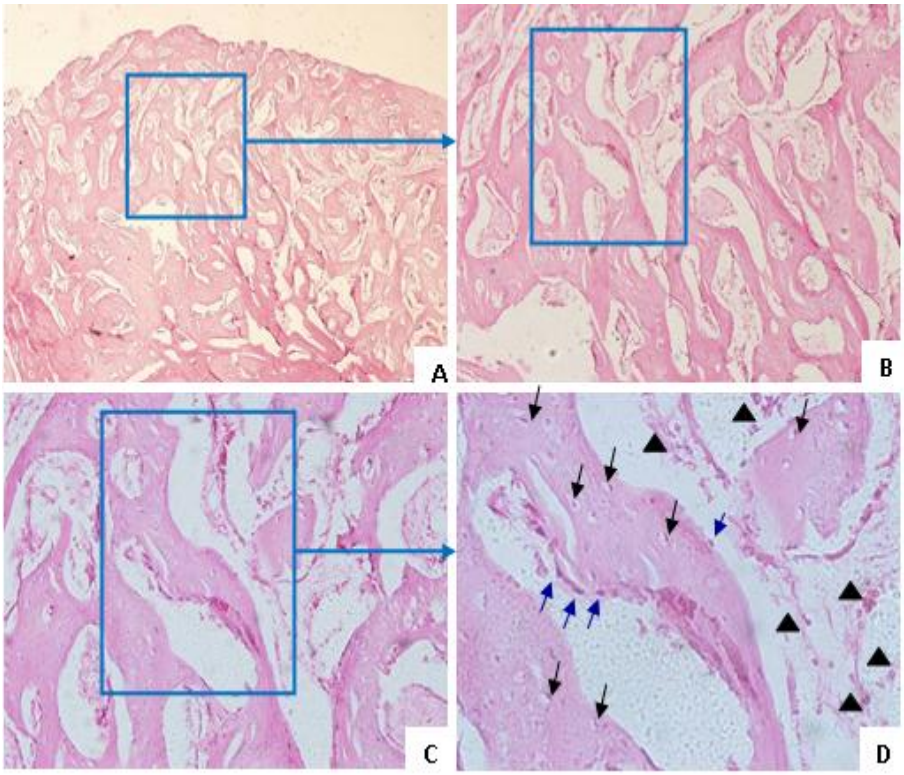

Figure 5. Evaluation results based on histological images for bone grafts at 1 month. (A). The structure of the bone graft after transplantation 1 month (× 4). (B) and (C). The structure of the bone graft was observed respectively in the objective lens (× 10) and (× 20). (D). The structure of the graft was observed in objective lens (× 40), indicating the presence of blood vessels within the bone graft (black triangles), osteocytes (black arrow head) and osteoblasts (blue arrow head).

When we compared the results of histological staining with the control group (only coral transplantation), the frame structure of the coral has not been stable due to coral frames have not yet transformed into skeletal structure has been break when performing histological staining (Figure 6).

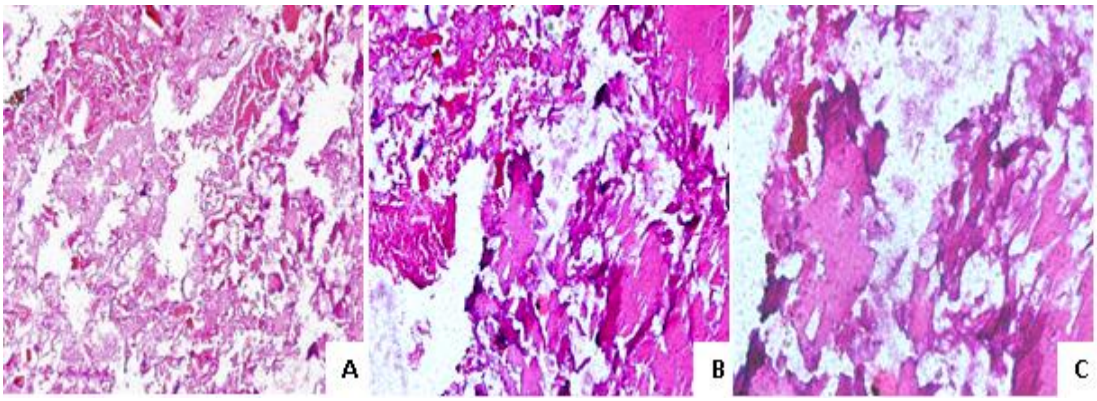

Figure 6. Results of histological evaluation in the control group at 1 month. (A). The structure of coral 1 month after transplantation (× 4). (B) and (C). The structure of the coral were observed respectively in the objective lens (× 10) and (× 20). The structure of the coral was not stable because the process of bone healing between bone tissue and the coral was not formed. Also on the coral has not happened the new bone tissue regeneration (not supplied by cells bone formation).

Results were grafted bone graft in rabbits at 3 months

After 3 months, the bone healing process was going well on the bone graft, bone graft has been adjusted gradually into new bone tissue (Figure 7). However, for the control group, the size of the coral is quite large and visible on the radiograph (Figure 7D, 7E). Histologic results in the control group also showed bone healing process occurs slowly and has been transformed into bone tissue.

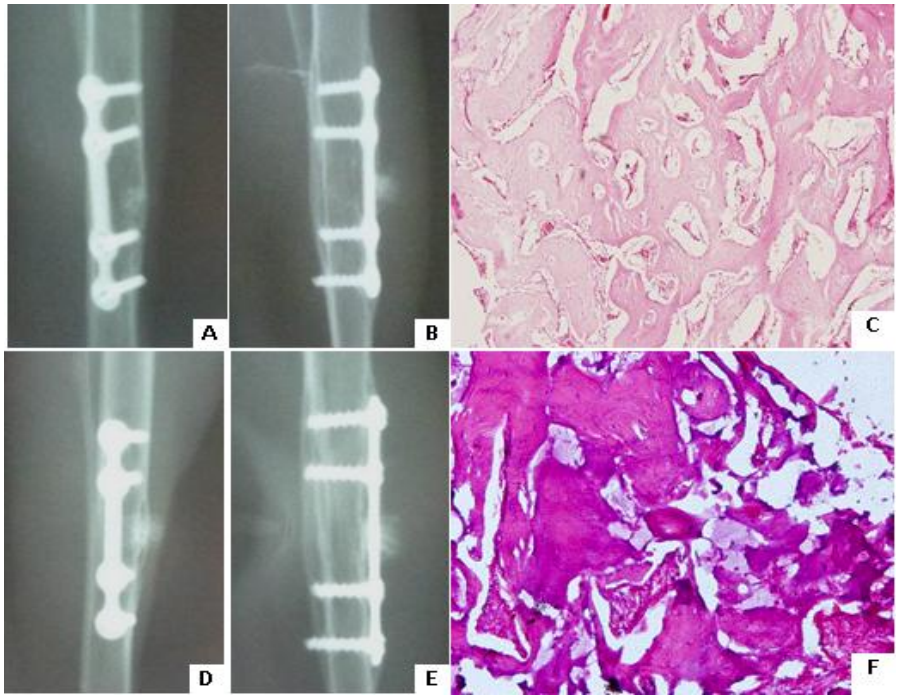

Figure 7. Results of research on rabbits at the time of three months. (A), (B). X-ray image of the bone fragments. (C). Histologic results of bone graft bearing (× 10). (FOR). X-ray images of the coral frame. (F). Histologic results of coral frame (× 10).

Results were grafted bone graft in rabbits at 6 months

After 6 months, the new bone tissue regeneration is almost completed when considering the results on the two research groups. However, when observed on histological results, there was a difference between the two study groups (Figure 8).

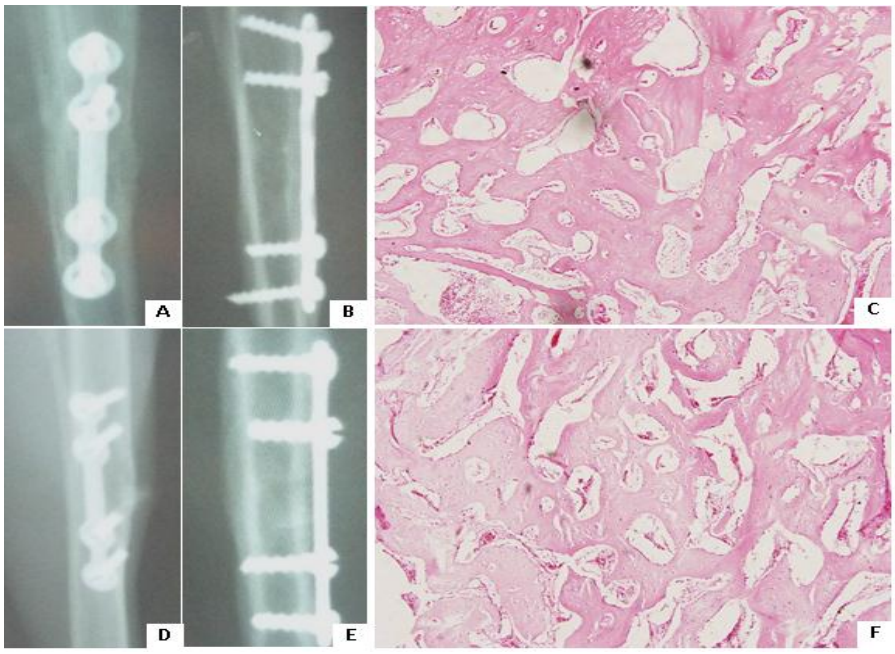

Figure 8. Results of research on rabbits at the time of six months. (A), (B). X-ray image of the bone fragments. (C). Histologic results of bone graft bearing (× 10). (FOR). X-ray images of the coral frame. (F). Histologic results of coral frame (× 10).

In study group, the process of bone tissue regeneration was completely finished, there was no difference when compared with the host bone tissue (Figure 8A, 8B). Quality of bone healing occurs evenly over all the structure of the bone graft (Figure 8C). The structure of the newly formed bone tissue is quite homogeneous and bone tissue cells present in that new bone tissue. When compared with controls, coral frames have been replaced almost entirely the host bone tissue. Inside the structure of bone tissue still appears much smaller bone cavities

and walls, which suggests that the process of bone regeneration has not been completed (also appeared a lot of new blood vessels within the framework of coral) (Figure 8F).

DISCUSSION

Mesenchymal stem cells have the potential to differentiate into osteoblasts on coral frame (shown by the enzyme alkaline phosphatase expression, one of the common markers were many authors have used to identify the osteoblasts.([4,6,7,11]). This is an important theoretical basis for our team instead of bone grafts generated from the combination of coral frame and mesenchymal stem cells. The osteoblasts are not only coral adhesive on the frame, but also the growth and development on the surface as well as inside the coral frame (Figure 2, 3).

When we compare the results in the two groups studied, the results showed that in the group of rabbits with transplanted grafts, the time for new bone tissue regeneration and healing of bone quality was better and faster the control group at 1 and 3 months. These results were demonstrated in specific x-ray image (Figure 4,7,8) and histologic images (Figure 4,7,8).

According to many researchers, after the period of 4-6 months, the bone graft has been completely replaced by host bone tissue when performed on rabbit models [1,5], in this study timelines, the comparisons between the team were very difficult. However, if based on histological image (picture), the regeneration and repair of bone in two experimental groups were not similar, while the team for bone healing pretty good results (Figure 8C), the results on the bone healing not as good as the control group (Figure 8F). This may be due to the source of mesenchymal stem cells existed on coral frame helped the new bone tissue regeneration happened better and faster. Also functions as scaffolding for bone formation (from coral frame), the bone graft had been adding elements to induce bone formation (from mesenchymal stem cells) from autologous bone marrow of rabbits. Thus, this bone graft can be evaluated equivalent to autologous bone grafts.

CONCLUSION

We noticed that the bone graft from coral frame containing autologous osteoblasts for bone healing faster results than the control group (only coral transplantation). This difference is due to the source of mesenchymal stem cells was added on coral frame. The results of this study opens a promising future to use the biological material supplemented with stem cell (from the patient) to create bone graft in order to meet the needs of bone graft to treatment of patients. This would be a great alternative to solve the problem of limited supply of human bone tissue.

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