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Study of osteocalcin and collagen type I in Regeneration of Atrophic Non-Union Fracture based on Bivalve Anodonta-PRP Formula and Mesenchymal Stem Cells as Composite Scaffold in Regeneration of Atrophic Non-Union Fracture

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Abstract: Atrophic non-union fracture is a further complication of a fracture in the form of non-bridging callus formation between the two fracture fragments accompanied by atrophy of the cells and tissue at the ends of the fragments and necrosis, thereby resulting in avascular defect. This complication is very difficult to manage and requires a operation of fixation and bone graft. Patients will experience a reduced quality of life due to the function of a limb is decreased and even reduced completely. The discovery of mesenchymal stem cells gives a promising for optimal treatment. Mesenchymal stem cells (MSCs) are adult stem cells produced by human bone marrow that will differentiate into osteoblasts. A vascular defect requires biomaterial (scaffold) and Platelet Rich Plasma (PRP). Scaffold is used to connect both ends of the defect, while the PRP is used to stimulate angiogenesis thereby resulting in neovascularization. This research used hidroxyapatite (HA) scaffold, which is used as an allograft, Which was from the scallops shells (*Bivalves anodonta*- BA). It produced biomaterial Chitosan, and it was expected to be new reported. This study used the post test only control group design with animals models of male New Zealand White Rabbit. The animals were divided into 3 groups (G1, G2, G3). Animal models were made by cutting the ulna bone and then cauterization on soft tissue and periosteum was made around the tip of the fracture. After 8 weeks, the defect was implanted with MSCs + PRP (G1), MSCs + PRP + Hidroxyapatite (G2), MSCs + PRP + BA (G3). Assay was conducted with immuno histochemistry for osteocalcin and collagen type I expression. The result of immuno histochemistry assay for expression of osteocalcin, collagen type 1 and histopathological assay showed that scaffold played very important role in the healing of atrophic non-union fracture that confirmed based on osteocalcin and collagen type I. In Conclusion, bone regeneration process was better in the groups implanted with MSCs + PRP + BA compared with the hydroxyapatite (HA) scaffold or without scaffold.

Keywords : Atrophic Non-Union Fracture, mesenshymal stem cells, Platelet Rich Plasma, bivalve Anodonta, collagen type I, osteocalcin, scaffolds.

Introduction

Cases of atrophic non-union fracture in the world are enormous. Therefore, it becomes one of the major problems in the health sector, especially in the field of orthopedics and traumatology. The causes are also very varied[1]. In Indonesia, it is difficult to obtain accurate data because many cases go unreported. In the rural areas or even in large cities, plenty of these cases are still handled by bone setter, resulting in non-union fractures. Atrophic non-union is very difficult to manage due to the a vascular condition at around defects. The management is very complex with surgery, fixation and implantation of vascularized bone graft (golden standard therapy)[2]. Conventionally, the bone graft derives from the patient's own bone (autograft). This surgical technique is very difficult so that the healing is not optimal and the limb function does not return to normal. There is a major drawback in autograft; it does not allow regeneration on the necrotic side of the bone due to the neovascularization process has not been proven[3], so that repeated fractures often occur due to the remodeling phase is not adequate[4]. Growth factor expression disorder also may cause conventional therapy[5].

Atrophic non-union fracture is the failure of the regeneration process in the fracture because the process stops in the inflammatory phase where the cause is the absence of vascularization on the defect occurring between the two ends of the fracture. In the fracture area, there is vascular endothelial damage which will activate the complement cascade, platelet aggregation and release of alpha granule. Platelet aggregation will express growth factors and cytokines and PMN, lymphocytes, monocytes and macrophages. Microenvironments that occurred in the area of the fracture are acidic PH, hypoxia and low O_2 pressure, so that the growth factors and cytokines failed in angiogenesis. In the microenvironment, the progenitor cells fail to proliferate and differentiate into osteoblasts, only proliferate and differentiate into chondroblasts and fibroblasts so that the fracture side experiences atrophy and no calcification[12].

Today administration of growth factors has been investigated on atrophic non-union fracture in rats, among others, by injection of bone marrow blood, as well as the administration of stem cell[7]. The administration of stem cells is intended for regeneration of necrotic bone.

Collagen type I is the resulting expression of the highest collagen (90%) of osteoblasts, where as osteocalcin is a non-specific collagen produced only by osteoblasts. Both collagens have essential role in the formaton ofthe matrixin the regeneration process. This study used Platelet Rich Plasma (PRP) extracted from venous blood of test animals. The PRP contains many growth factors, among others, BMP2, TGFβ, IGF-1, FGF2, PDGF, KGF, IL8 and most importantly there is VEGF, which stimulates the neovascularization[8].

The rapid development in the function of stem cells is still far from perfect, particularly on bone tissue in the form of hard tissue.In the bone tissue, the stem cells cannot stand alone. A construction is needed as a buffer or scaffold between the bone tissues. Buffer or scaffold construction is conventionally in the form of graft[9,10]. In this study,the scaffold was selected from shells (bivalves Anodonta) in accordance with the requirements of bone material, where they belong to natural biopolymers containing Chitin-proteins to demineralization and deproteinization process into Chitosan compound. Some authors have been conducting researches using hydroxiapatite with very promising results and is regarded as the golden standard graft.Therefore,this study used hydroxiapatite (HA) as a control.

Material and Method

The study was carried out in the Biomaterial Center and Tissue Bank of General Hospital Dr. Soetomo, Institute of Tropical Disease (ITD), Airlangga University (Unair), Electron Microscope Laboratory of Unair, Anatomic Pathology Laboratory Unair Surabaya

Animal models:

NewZealand White Rabbit, male, aged of 6-9 months, body weight 3-4 kg. The exclusion criteria stated that animals with other injuries and infections were excluded from the study. Drop out criteria included surgical wound infections and death. Model animals were prepared as follws: rabbits were anesthetized, then the os ulna at the ⅓ right middle was cut, and further circular soft tissues were cauterized on both ends of bone,each 1mm (1). After 8 weeks the test animals were divided into 3 groups, consisting of treatment group1 (G1), defects implanted with MSCs+PRP; treatment group 2(G2), defects implanted with MSCs+PRP+HA; and treatment

group 3 (G3), defects implanted with MSCs+PRP+bivalvesAnodonta (BA). Doses of scaffold were Chitosan powder=50µg, MSCs=2x10^6, PRP=80% by volume of the defect, HA=50µg.

Research Procedures:

The research was carried out by several steps, such as obtaining an Ethical Clearance of the Ethics Committee of UB Medical faculty. Having obtained the Ethical Clearance,the next steps were as follows: (1). Bone marrows were aspirated in test animals atthe trochanter femur region of 5 cc. (2). Isolation, culture and expansion of BM-MSCs. Mononuclear cells were separated with Ficoll-Hyupaque density gradient. (3). BM-MSCs were characterized with immunohistochemical assay using antihuman monoclonal antibodies FITC CD45 and CD105. (4). Preparation and characterization of Bivalve Anodonta-Chitosan. Biomaterials, in which the shells were acetylated and characterized byJEOL JSM-T100 Scanning Microscope. (5). MTTAssay. (6). Formulation of composite MSCs, scaffold and PRP. (7). Preparation of test animal model. (8) Implantation of composites into Rabbit as animal models. (9). Decalcification with EDTA liquid.

Data Assessment:

After 16 weeks of decalcification, then the data were assessed as follows: (1). Immunohistochemistry assay to measurethe thickness of collagen type I by using themo use monoclonal antibody to collagen type I. (2). Immunohistochemistry assay for counting the number of positive osteocalcin using mouse monoclonal anti-osteocalcin antibody.

Results

Osteocalcine Expression on Osteoblas of Atrophic Non-Union Fracture

Mesenchymal Stem Cells(MSCs) were obtained from the bone marrow of the test animals which were isolated, cultured, expanded and characterized using the Ficoll density gradient. The researchers used CD105 markers for positive MSCs, and CD45 for negative MSCs. The number of cells used was 2x10^6 on the differentiated cells. PRP was prepared from venous blood of the test animals at 20ml and then centrifuged at 3000 rpm for 13 min and 30,000 rpm for 15 min and produced approximately 2ml high concentration of platelets.

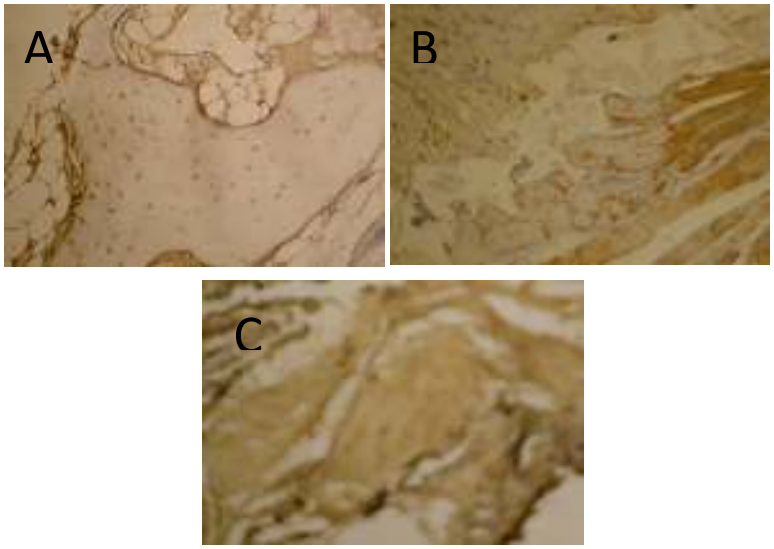

Figure 1. Osteocalcine expresion of osteoblast on Atrophic Non-Uion Fracture by Immunohistochemistry Technique.

Note : A : G1 (MSCs + PRP
B : G2 (MSCs + PRP + Hidroxyapatite
C: G3 (MSCs + PRP + BA

Both treated group of G2 and G3 showed the increasing of Osteocalcine expression of osteoblast in Atrophic non-union fracture. Comparison of the percentage of osteoblasts expressing osteocalcin in three treatment groupsG1, G2 and G3 showed in Fig 2.

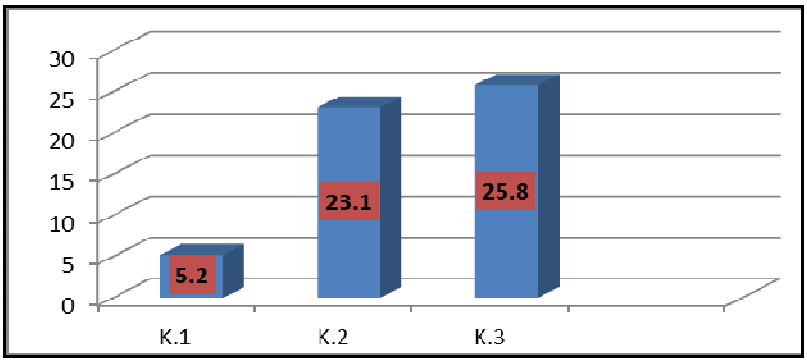

Figure 2. Profil of osteoblast cells with positive osteocalcin

Note : K.1 :G1 (MSCs + PRP
K.2: G2 (MSCs + PRP + Hidroxyapatite
K.3: G3 (MSCs + PRP + BA

Regeneration of atrophic non-union fracture was assessed after 8 weeks post-implantation of MSCs + PRP (G1), MSCs + PRP + HA (G2), and MSCs + PRP + BA (G3)

Osteocalcine Expression on Osteoblas of Atrophic Non-Union Fracture

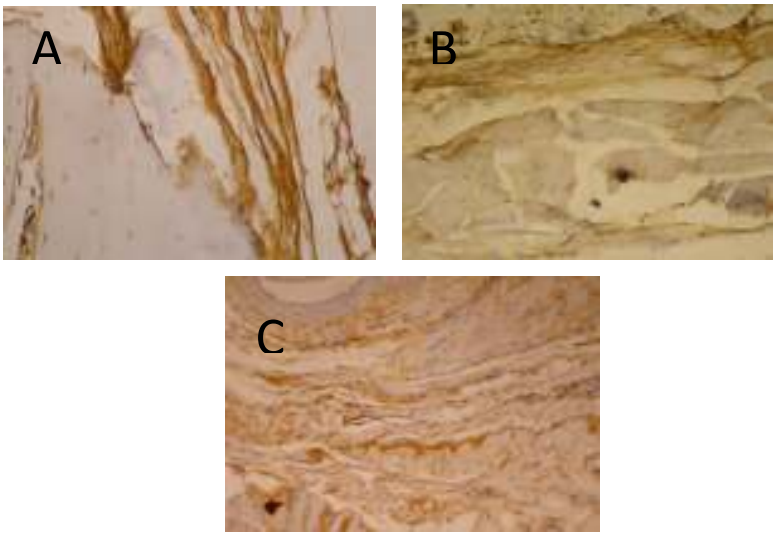

Figure 3. Collagen Type 1 expresion of osteoblast on Atrophic Non-Union Fracture by Immunohistochemistry Technique.

Note : A : G1 (MSCs + PRP
B : G2 (MSCs + PRP + Hidroxyapatite
C: G3 (MSCs + PRP + BA

Hidroxyapatite (HA) and *Bivalves anodonta* (BA) increase Collagen Type 1 of osteoblast on Atrophic Non-Union Fracture (Fig. 4)

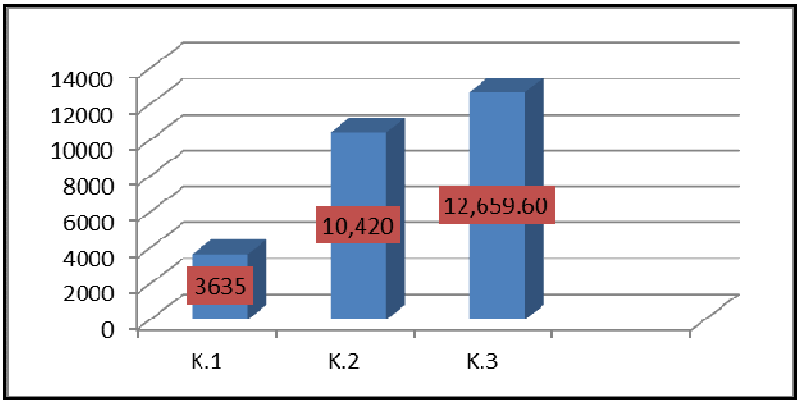

Figure 4. Profil of osteoblast cells with positive Collagen Type 1.

Note : K.1 :G1 (MSCs + PRP)
K.2: G2 (MSCs + PRP + Hidroxyapatite)
K.3: G3 (MSCs + PRP + BA)

Discussion

This study indicated that the scaffold plays very important role in the healing of atrophic non-union fracture. Conventional therapy in this case was carried out by the implantation of vascularized bone graft in the defect. Autograft often results in morbidity and the results achieved are also less satisfactory. Then allograft technique was developed with a variety of biomaterials under study. Hidroxyapatite is considered as a promising scaffold and is regarded as the golden standard theraphy until today[10].

This research used Chitosan from Bivalves Anodonta as alternative graft that was expected to bemore promising and beneficial compared with hydroxyapatite[4,11]. Bivalve Anodonta Chitosan as scaffold of Mesenchymal Stem Cells is a composite that is eligible for the regeneration of fracture[12]. The shell-derived Chitosan is a natural scaffold resulting from chitin proteins undergoing demineralization, deproteinization and then deacetalization into active Chitosan compounds[13]. In the case of atrophic non-union fracture, the researchers added PRP as a growth factor to generate more perfect neovascularization[14,15].

Mesenchymal Stem Cells (MSCs) were obtained from the bone marrow of the test animals which were isolated, cultured, expanded and characterized using the Ficoll density gradient. The researchers used CD105 markers for positive MSCs, and CD45 for negative MSCs. The number of cells used was $2x10^6$ on the differentiated cells[16].

Platelet Rich Plasma (PRP) was prepared from venous blood of the test animals at 20 ml and then centrifuged at 3000 rpm for 13 min and 30,000 rpm for 15 min and produced approximately 2 ml high concentration of platelets.

Regeneration of atrophic non-union fracture was assessed after 8 weeks post-implantation of MSCs + PRP (G1), MSCs + PRP + HA (G2), and MSCs + PRP + BA (G3). In the G1, MSCs generating osteoblast cells had a function as osteogenesis. PRP will release a growth factor that serves as osteoconduction. MSCs and PRP take role in the proliferation and differentiation into fibroblasts, condroblast and osteoblasts so that at reparative phase the osteoblast cells will produce new bone matrix. However, the bone matrix formed only occupies certain areas and did not connect area between the two sides of the bones that make up the defect. Bridging matrix did not form due to the absence of scaffold that serves as a bridging construction. In the G2, at the reparative phase the osteoblast cells produced bone matrix and the hidroxyapatite scaffold might serve as osteoconductive, allowing a formation of bridging matrix that fills the defect. At the G3, bivalve chitosan Anodonta scaffold with formula of MSCs and PRP could produce better bridging matrix than G2. The number of osteocalcin were calculated as: G1 = 16.52%, G2 = 36.52%, and G3 = 37.78%.

Conclusion

The results of the study showed that the number of osteocalcin expressed by osteoblast was the highest

in the treatment of group 3. Collagens type I was the thickest in the treatment of group 3. The matrix area was the most extensive in the treatment group 3; thus it can be concluded that the regeneration of atrophic non-union fracture achieved the best outcome with the formula of bivalve Anodonta-PRP and MSCs as a scaffold composite.

References

1. Sfeir C, Ho L, Doll BA. 2005. Fracture Repair. In (Lieberman JR, Friedlander GE, eds). *Bone Regeneration and Repair, Biology and Clinical Application.* Humana Press, Totowa. New Jersey. 21-44.
2. Kokobu T, Hak DJ,Hazelwood SJ,Reddi AH. 2008. Development an atrophic non union model and comparison to a closed healing fracture in rat femur. *J orthop Res*, 21: 503-510.
3. Delloye C, Cornu O, Druez V. 2007. Bone Allogratt What They Can Offer and What They Cannot. *J. Bone Joint Surg (Br),* Vol 89-B: 574-579.
4. Hernigou PH, Poignard A, Beaujean F, Rouard H, 2005. Percutaneous Autologous Bone-Marrow Grafing For Nonunions; Influence of the Number and Concentration of Progenitor Cells. *J. Bone Joint Surg*; Vol 86-A(7): 1430-1437.
5. Brownlow HC, Reed A, Simpson AH. 2001. Growth factor expression during the development of atrophic non union. *Injury*.; 32: 519-524.
6. Reed AA, Joyner CJ, Isefuku S, Brownlow HC, Simpson AHRW. 2008. Vascularity in a new model atrophic non union. *J Bone Surg Br*. 85:604-610.
7. Minamide, Nakamoto C. 2011. Engineering Principles of Clinical Cell-Based Tissue Engineering. *J. Bone Joint Surg*; Vol. 86-A(7):1542-1558.
8. Day SM, Ostrum EF, Chao RF, 2000. *Bone Injury, Regeneration, and Repair*. In (Buckwalter JA, Eionhorn TA, Simon SR, eds). Orthopedic Basic Science Biology and Biomechanic of the Musculoskeletal. System. 2nd ed American Academy of Orthopaedic Surgeon. 371-400.
9. Rumpel E, Wolf E and Kauschke E, 2006. The Biodegradation of Hydroxyapatite Bone Graf Substitutes In Vivo. *Folia Morphol,* 65(1); 43-48.
10. Goldstein AS, 2007. *Cell Adhesion. In Tissue engineering* (Edited by Fisher JP, Mikos AG, Bronzino JD). CRC Press, Boca Raton: 5-t-5-17.
11. Yamada Y, Ueda M, Naiki T, 2004. Autogenous Injectable for Regeneration with mesenchymal stem cells and platelet rich plasma: Tissue Engineering Bone Regeneration. Tissue Eng Vol.10(5/6): 688-698.
12. Moucha CS, Einrohn TA, 2005. *Bone Morphogenetic Proteins and Other Growth Factors to Enchance Fracture Healing and Treatment of Nonunions*. In (Lieberman JR, Friedlander GE, eds). Bone Regenaration and Repair, Biology and Clinical Application Humana Press, Towota. New Jersey. 169-194.
13. Yao KD , Zhao F, 2002. *Chitosan based gels*, edited by M Schwartz, pp 182-190.
14. Dutta PK, Ravikumar MNV and Dutta J. 2002. Chitin and chitosan for versatile applications, *JMS Polym Rev*, C 42.
15. Yen M Yang J, Mau J. Physicochemical characterization of chitin and chitosan from crab shells. *Carbohydr Polym*; 75: 15-21
16. Di Martino A,Sittinger M, Risbud MV (2005) Chitosan: a versatile biopolymer for orthopaedic tissue-engneering. *Biomaterials* 26 : 5983-90.

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