Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 20 (December Suppl.) : 2018 : S134-S137

ISSN-0972-3005

EXPRESSION OF TRANSFORMING GROWTH FACTOR-β1 AND OSTEOCALCIN IN RAT CALVARIA DEFECT AFTER APPLICATION OF BOVINE CORTICAL BONE MEMBRANE

NURUL FARIZAH[1], DAVID B KAMADJAJA[2,4]*, ADELINE WIBOWO[1], PRATIWI SOESILOWATI[3] AND ACHMAD HARIJADI[2]

[1]Residency Program, Department of Oral and Maxillofacial Surgery, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia
[2] Department of Oral Maxillofacial Surgery, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia
[3] Department of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia
[4] Stem Cell Research and Development Center, Universitas Airlangga, Surabaya, Indonesia

(Received 25 September, 2018; accepted 15 November, 2018)

Key word: *Bovine cortical bone membrane, Calvaria defect, TGF-β1, Osteocalcin*

Abstract– Bovine pericardium collagen membran (BPCM) had been widely used in guided bone regeneration (GBR) whose manufacturing process usually required chemical cross-linking to prolong its biodegradation. A newly developed bovine cortical bone membrane (BCBM)is rich with growth factors which theoretically has osteoinductive capacity. It has been proven biocompatible, however its osteoinduction potential still needs to be revealed. This study evaluated the osteoinductive potential of BCBM compared with BPCM applied in critical-sized defects of rat's calvaria. Critical sized defect was made on calvaria bone of 30 rats. The samples were divided into 3 groups, each contains of 10 rats. The defects were then covered by BCBM dan BPCM in the first two groups. The third group was control group, without membran application. Samples were sacrificed at 2 and 4 weeks. Expressions of TGF-β1 and osteocalcin were examined using immunohistochemistry (IHC) method and the data analyzed statistically with the p value < 0.05 being significantly different. TGF-β1 expression was insignificantly different between BCBM and BPCM groups. Meanwhile, the osteocalcin expression was significantly higher inBCBM compared to BPCM group ($p < 0.05$) through 2 and 4 weeks of observation. Bovine cortical bone membrane has similar osteoinductive potential to BPCM based on the expression of TGF-β1, but has better bone healing result based on osteocalcin expression. Therefore it is potential to be used as GBR membrane.

INTRODUCTION

Reconstruction of alveolar bone defect required bone grafting procedure (Retzepi and Donos, 2010). However, to improve the bone regeneration it was important to keep the grafted defect separated from fibrous organization by inserting membranes following the principle of guided bone regeneration. Most commonly used GBR membrane is bovine pericardium membrane (BPCM) however it associated with prolonged degradation rate which cause poor tissue integration (Bunyaratavej and Wang, 2001).

Bovine Cortical Bone Membrane (BCBM), a novel GBR membrane, isobtained from demineralized bovine cortical bone. This BCBM is expected to be an alternativemembrane for GBR procedure because theoretically it has osteoinductive potential (de Oliviera *et al*., 2004).

In the process of bone healing, TGF-ß1 increases osteoblast proliferation, prevents apoptosis of osteoblasts, and recruits osteoblast precursors or matrix-producing osteoblasts to place the defect through chemotactic attraction. Osteocalcin is a non-collagen protein hormone secreted by osteoblasts. High levels of osteocalcin indicate high bone density (Cantatore, 2004). In this study, an analysis of the direct effects of bovine cortical bone membrane on bone regeneration will be carried out, assessed by TGF-β1 expression and expression of osteocalcin which describes the process of osteoblast differentiation in bone regeneration process.

***Corresponding author's email**: david-b-k@fkg.unair.ac.id

MATERIALS AND METHODS

Study design

This research is Post Test Only Control Group Design with observation of TGF-β1 and osteocalcin expression inrat's calvarial bone defect histologically after implantation of BCBM and BPCM in 2nd and 4th week. The samples were divided into 3 groups, with 10 each sample in one group. In the first group BCBM application was performed on calvaria bone defect, second group was carried out BPCM application, and the third group was left without membrane application (control group).

Surgical Procedure

This study was conducted on experimental animals 30 Wistar strain rats aged 10-12 weeks with a weight of 120-140 grams. The procedure begins with intramuscular injection of ketamine HCl (20 mg / kg body weight). After the anesthetic has worked, the area of the dorsum region is shaved and povidone iodine disinfection is 10%. Skin incisions were performed on the rat's head in the medial section, then mucoperiosteal flap retraction was performed until calvaria bone was obtained. Medial suture was identified in calvaria rats. The defect was made on the right lateral side of the medial suture using a bur of 5 mm in diameter as thick as 0.8 mm. The membrane measuring 1x1 cm was applied over the defect until the defect is completely covered by the membrane. DFDBCB membrane (Tissue Bank, Dr. Soetomo Hospital) and BPCM (Batan, Indonesia) were used in experimental and positive control groups, respectively, while no membrane was applied in negative control group. Then suturing the incision wound on the skin with silk suture 3-0. After the second and fourth week after application, 5 animals per group were sacrificed, to obtain sample for IHC examination.

Histology and immunohistochemistry examination

Tissue specimens which have been cut are soaked in 10% buffered formaldehyde fixation solution, at least 24 hours before the decalcification process is carried out. Specimen decalcification is carried out with EDTA solution for 30-40 days. Subsequently, all the specimens were processed under routine procedure for histology (HE staining). For immunohistochemical staining mousemonoclonal antibody towards TGF-β1 and Osteocalcin are employed

Observation and Calculation of TGF-β1 Expressions and Osteocalcin

The observations were carried out using a 400x magnification light microscope. Each preparation was examined in five fields of view and the value of each field of view was calculated by the number of glowing osteoblasts to obtain TGF-β1 expression values osteocalcin quantitatively. Observations were carried out by 3 reviewers. The results of observations from 3 reviewers are summed, then the average value is taken so that the expression value of TGF-β1 danosteocalcin is obtained (number of glowing osteoblast cells).

The data from calculation of the parameters above is statistically analyzed with one way Anova and post hoc Tukey HSD. Significant difference is determined with p value < 0.05

RESULTS

The observations and the measurements of TGF-β1 and osteocalcin expressionwas performed in the three research groups: (1) BCBM treatment group; (2) BPCM treatment group; (3) control group (without membrane), with each sample observed in the second and fourth week. The result of immunohistochemical staining on osteoblast with TGF-β1 and osteocalcin antibodies are provided in Figure 1.

From the diagram in figure 2, it is shown thatin the second week the mean expressionof TGF-ß1 in BCBM group was higher than in BPCM and control group. Whereas in the fourth week, the mean expression of TGF-β1 in BPCM group was higher than the BCBM group and the control group.

Based on the results of the ANOVA test ofTGF-ß1 expression, it was shownthat there was no significant difference ($p< 0.05$) in BCBM, BPCM, and control group on 2nd week. Meanwhile on 4th week showed there was significant difference ($p<0.0001$) in the 3 groups. Multiple comparison test showed significant difference between control group either with BCBM or BPCM group.

From the diagram in Figure 2, it is shown thatin the second week the mean expression of osteocalcin in BCBM group was slightly higher than in BPCM but much higher than control group. Whereas in the fourth week, the mean expression of osteocalcin in BCBM group was much higher than the BPCM group and the control group. Based on the results of the ANOVA test ofosteocalcin expression, statistical analysis showed there was significant difference

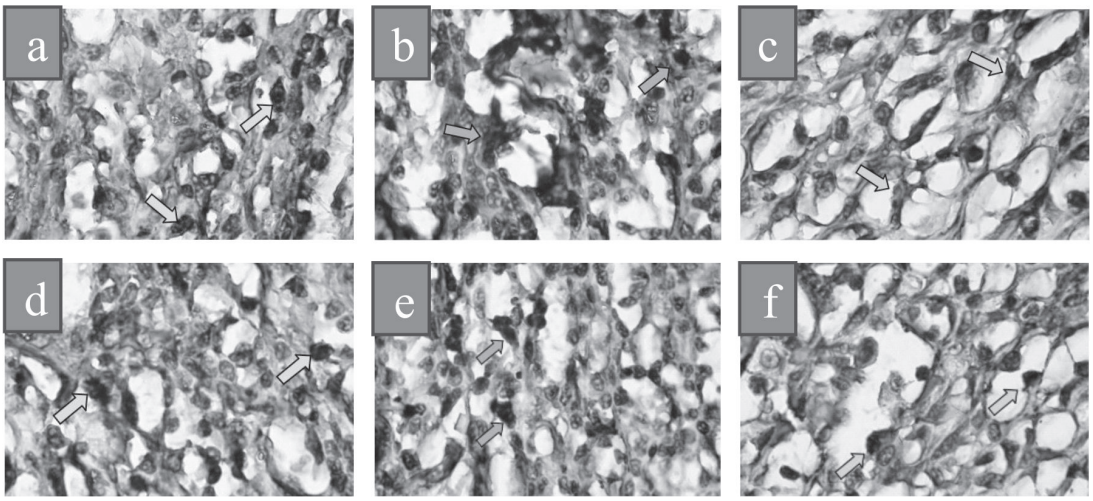

Fig. 1. Expression TGF-ß1 and osteocalcin 2 weeks post implantation of BCBM and BPCM. (a, b, c) shows the TGF-ß1 expressing cells in BCBM, BPCM, and control group (d, e, f)shows the osteocalcin expressing cells in BCBM, BPCM, and control group. (arrows pointing to cells positively stained with their respective antibodies)

($p<0.05$) in BCBM, BPCM, and control group on 2nd and 4th week.

Multiple comparison test revealed significant differencebetween BCBM and BPCM groups and

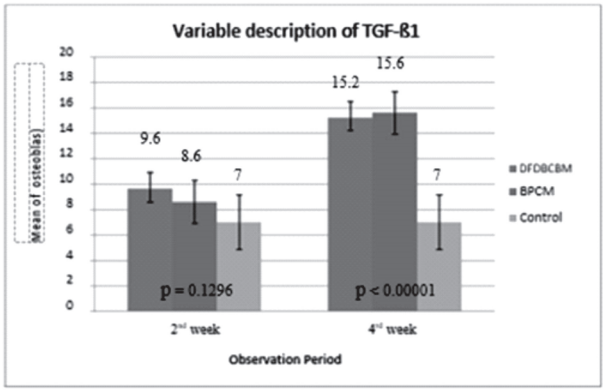

Fig. 2. Mean ofTGF-ß1 expressionin BCBM, BPCM and control group in 2nd and 4th week

control group on second week, whereas on fourth week significant differences were found between BCBM and BPCM group compared to control group as well as between BCBM and BPCM group.

DISCUSSION

From the results showed that insignificant difference in TGF-β1 expression for post application of BCBM and BPCM in the second and fourth week. TGF-β1 is mainly produced in the initial phase of the bone healing process, and will slowly decrease

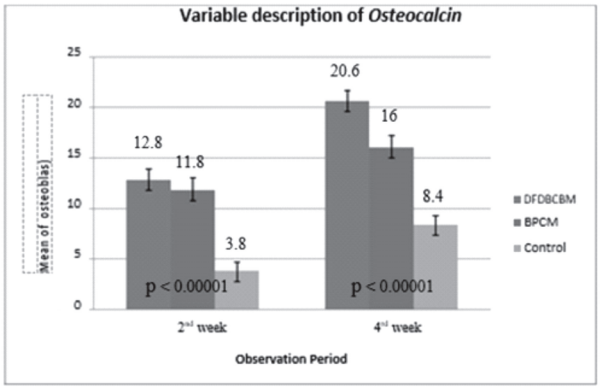

Fig. 3. Average osteocalcinexpression in BCBM group, BPCM group, and control group in 2nd and 4th week

but continue to be produced during the healing process. In this study, TGF-β1 expression was measured only from TGF-β1 which had bind to receptors on osteoblast cells, not the overall number of TGF-β1 expressed by cells in the area of bone defects (Filvaroff, 1999).

In the second week, TGF-β1 was mainly produced by chronic inflammatory cells and fibroblast cells in the defect area. Therefore there was no significant difference in the number of TGF-â1 binding to osteoblast cells between the BCBM group and the BPCM and control groups. In the fourth week, TGF-β1 originates primarily from osteoblast cells. Increasing TGF-β1 expression shows that the number of osteoblast cells expressing TGF-β1 is increasing. The presence of TGF-β1

expression by osteoblasts in the BCBM and BPCM groups shows that both membranes have osteoinduction ability. And the absence of significant differences in TGF-β1 expression between BCBM and BPCM groups showed that both membranes have equivalent osteoinduction potential.

The results of osteocalcin expression after BCBM application were higher than the BPCM and control groups in the second week. In which there is insignificant difference between the BCBM and BPCM groups, while between the BCBM group and the control group there are significant differences. High expression of osteocalcin indicates the number of adult osteoblast cells in the area of bone defects.

In the fourth week, osteocalcin expression in the BCBM group was significantly higher than in the BPCM and control groups. The number of adult osteoblasts in the BCBM group at fourth week can occur due to the stimulation of growth factors that increase the proliferation and differentiation of osteoblasts, so that the bone regeneration process occurs better in the defect applied by BCBM membrane. Where the results of the process are marked by the high number of adult osteoblasts in the fourth week (Yang, 2012).

CONCLUSION

Bovine cortical bone membrane has similar osteoinductive potential to BPCM based on the expression of TGF-β1, but has better bone healing result based on osteocalcin expression. Therefore it is potential to be used as guided bone regeneration membrane.

REFERENCES

Bunyaratavej, P. and Wang, H. 2001. Collagen membranes: a review. *Journal of Periodontology.* 72 (2) : 215-229

Cantatore, F.P., Corrado, A., Grano, M., Quarta, L., Colucci, S. and Melillo, N. 2004. *Osteocalcin Synthesis by Human Osteoblasts from Normal and Osteoarthritic Bone after Vitamin D3 Stimulation*. *Clin Rheumatol*. 23 (6) : 490-495.

Liu, J. and Kerns, D.G. 2014. Mechanisms of Guided Bone Regeneration: A Review. *The Open Dentistry Journal*. 8 (Suppl 1-M3) : 56-65.

Retzepi, M. and Donos, N. 2010. Guided bone regeneration: biological principle and therapeutic applications. *Clinical Oral Implants Research*. 21 (6) : 567–576.

de Oliveira, R.C., Menezes, R., Cestari, T.M., Taga, E.M., Taga, R.,Buzalaf, M.A.R. and Granjeiro, J.M. 2004. Tissue Response to a Membrane ofDemineralized Bovine Cortical Bone Implanted in The Subcutaneous Tissue of Rats. *Brazilian Dental Journal*. 15 (1) : 3–8.

Yang, Y.Q., Chai, L., Li, X.T., Zhang, D. and Rabie, A. 2012. *OsteocalcinExpression in Cells: Recent Research in Denstistry.* Nova Science Publishers, Inc.

Zhang, Y., Zhang, X., Shi, B. and Miron, R.J. 2013. Membranes for guided tissueand bone regeneration. *Annals of Oral & Maxillofacial Surgery.* 1 (1) : 10.