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ANALYSIS OF TISSUE RESPONSE AFTER SUBCUTANEOUS IMPLANTATION OF DEMINERALIZED FREEZE-DRIED BOVINE CORTICAL BONE MEMBRANE

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***Key words**: Demineralized freeze-dried bovine cortical Bone membrane, Fibroblast capsule, Fibrous capsule, Interface tissue*

Abstract– Guided bone regeneration (GBR) for alveolar bone augmentation commonly uses collagen membrane made from bovine pericardium membrane (BPCM). However, it has been associated with prolonged biodegradation. Due to that reason, an innovation is needed to manufacture an alternative of GBR membrane which is demineralized freeze-dried bovine cortical bone membrane (DFDBCBM). However, its biocompatibility needs to be revealed by evaluating tissue response after DFDBCBM implantation. This study aims to analyze tissue response to DFDBCBM after subcutaneous implanted in rat's dorsum compared with that of BPCM. This study used 32 samples of rat divided into 2 groups (DFDBCBM and BPCM). Samples from each group were sacrificed after 7, 14, 21, and 28 days of subcutaneous implantation. The specimens were processed and stained with Hematoxylin-Eosin for histology examination to evaluate the thickness of the fibroblast capsule and the quality of the fibrous capsule as well as interface tissue. Data collected was statistically analyzed with p value of < 0.05. This study showed no statistically difference in the quantity of fibroblast capsule and quality of fibrous capsule as well as interface tissue between DFDBCBM group and BPCM group ($p > 0.05$). Demineralized freeze-dried bovine cortical bone membrane elicits similar tissue response compared to bovine pericardium membrane. The result indicates that DFDBCBM is a potential alternative for guided bone regeneration membrane.

INTRODUCTION

Bone graft is one method used to repair a bone defect due to any pathologic condition, trauma, infection, physiologic condition or congenital deformity (Torres *et al.,* 2014). 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 (Buser, 2009). *Guided Bone Regeneration* (GBR) is a procedure that allow any bone tissue to grow in a space covered by a tissue barrier. The biomaterials used in GBR should meet criteria such as has a biocompatible property, cell occlusive, reacted with the host tissue, and space making.Collagen from bovine pericardium had been widely used as resorbable membranes material because of its biocompatibility, hemostatic activity, and tissue integration. As a type of native collagen, bovine pericardium collagen could be rapidly resorbed; therefore its manufacturing process usually involved chemical cross-linking to prolong its biodegradation. However cross-linking process of the collagen fibrils was associated with poorer tissue integration and delayed vascular invasion. In addition, an increased invasion of

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inflammatory cells had been observed after implantation of chemically cross-linked collagen

Demineralized Freeze Dried Bovine Cortical Bone Membran (DFDBCBM) is a collagen membrane isolate from the bovine bone cortex. DFDBCBM is a potential material that can be used as GBR materials, it was important to determine that it was biocompatible, which meant that it should not cause antigenicitycytotoxicity, and excessive immune response. Besides, in order to be clinically effective as a barrier membrane it should not cause abnormal tissue response or undergo too early degradation. This study was aimed to analyze tissue response after implantation of DFDBCB membrane.

MATERIALS AND METHODS

DFDBCB Membrane Manufacturing Process

DFDBCBM processing was performed at Tissue Bank/Center for Biomaterial and Stem Cell, Dr. Soetomo General Hospital, Surabaya, as follows. Bovine cortical bone was immersed in 3% hydrogen peroxide solution to remove blood, fat, and bone marrow. The solution was replaced daily until the bone turned white and no trace of fat and marrow was detected after which the bone was washed out by soaking in daily replaced, sterile distilled water for 5 to 6 days. The cortical bone was then cut up into pieces with band saw under sterile condition. Demineralization was performed by immersing the bone in 0.1% HCL solution until the desired flexibility of the bone was achieved. The excess of HCL was subsequently washed out by soaking the "soft bone" in sterile distilled water many times until neutral pH was achieved, checked with pH meter. The demineralized bone was then cut into layers of membrane with 300 5m thickness using special microtome. Freeze drying was done by freezing for at least 24 hours and subsequently dried for 18–24 hours until less than 5% water content was achieved, followed by double packaging and sterilization using gamma irradiation.

This is an experimental study with post test only group design. This study conducted to evaluate the quantity of fibrous capsule, quality of fibrous capsule, and quantity of tissue interface on wistar rats after the implantation of *Demineralized Freeze-Dried Bovine Cortical Bone Membran* (DFDBCBM) as the experimental group and using pericardium membrane as the control group in vivo medium after day 7, 14, 21, and 28.

Tissue Response and Biodegradation Evaluation. Forty male Wistar rats used in this study were randomly divided into 2 groups. A 5 × 5 mm BPCM (Jason Membrane, Botiss, Germany) and DFDBCBM were subcutaneously implanted in rat's dorsum as control and experimental group, respectively. Five samples from each group were sacrificed at 7, 14, 21, and 28 days after implantation for histology examination followed by histomorphometry analysis to evaluate tissue response and biodegradation behavior of the membranes. The specimen are observed for fibrous capsule, quality of fibrous capsule, and the quality of tissue interface under the microscope. Group statistical analysis was performed with Mann Whitney test using SPSS.

RESULTS

Examination of the histological sections revealed a characteristic and some what uniform tissue response without signs of prolonged inflammatory reaction in BPCM and DFDBCBM groups. Fibrous capsule surrounding the membranes was from few cells thick in the initial phase of healing (day 7) to approximately 20–30 cells thick in later healing stage (Figure 1). The capsule contained more fibroblasts as primary cellular component in early phase but turned to be more fibrotic, with few fibrocytes, in later stage indicating maturity of the capsules. The capsules, in some area of the membranes, made direct contact with the membrane surface without the presence of layers of reactive cells but in majority there existed layers of fibrous capsules containing macrophages and foreign body giant cells which in this study was referred to as interface tissue (Figure 1).

The data of fibrous layer quantification showed that the median score of DFDBCBM group was higher than that of BPCM group in early phase (day 7) and late phase (day 28) (Figure 2); however there was no statistical difference in the observed variable (5]> 0.05) between the two groups.

The data of fibrous layer quality showed that the median score of both BPCM and DFDBCBM group showed upward trend from early phase towards intermediate and late phase of healing (Figure 2). Statistical analysis showed that there was no difference in the observed variable (5]> 0.05) between the two groups.

The data of fibrous layer quality showed that the

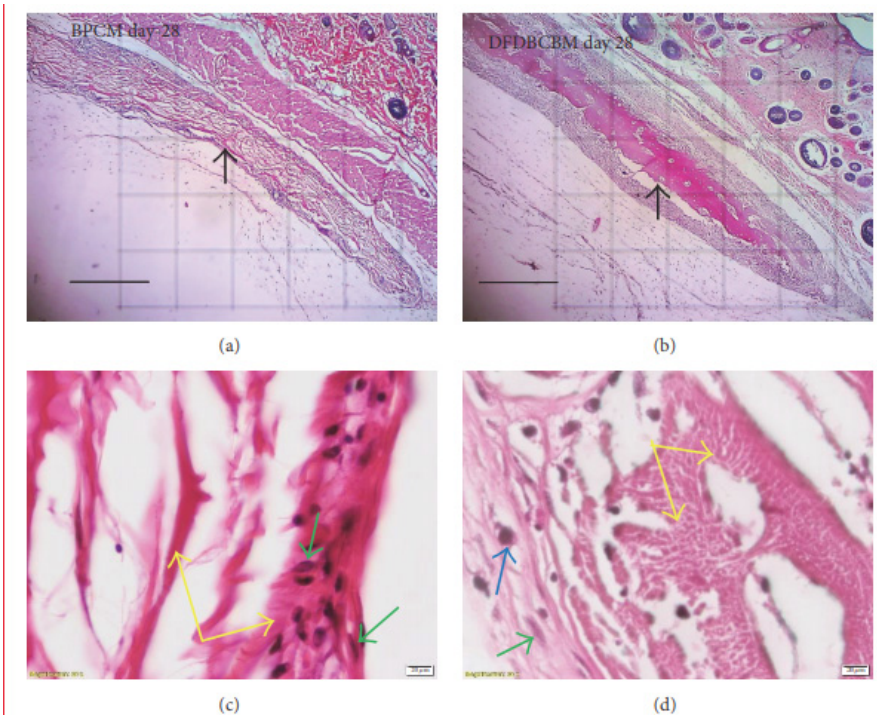

Fig. 2. Analysis of tissue response following subcutaneous implantation of BPCM and DFDBCBM. There was no significant difference in capsule quantity (a), capsule quality (b), and interface quality (c) between the two groups throughout the observed healing periods.

median score of both BPCM and DFDBCBM group showed upward trend from early phase towards intermediate and late phase of healing (Figure 2). Statistical analysis showed that there was no difference in the observed variable ($p>0.05$) between the two groups.

DISCUSSION

The tissue response analysis is done by measure the quantity of fibroblast capsule, quality of fibrous capsule, and quality of tissue interface. The quality of fibrous capsule indicate any proliferations that occur after implantation of the membrane. Normal tissue response to implantation of biomaterial followed physiologic process of healing which consisted of cellular infiltration, release of chemokines from cells (1–5 days), recruitment of tissue repair cells (5–15 days), and fibrous encapsulation and granulation tissue formation (3-4 weeks) (Anderson, 2008). After the resolution of acute and chronic inflammatory responses had occurred, granulation tissue was seen and confirmed by the presence of macrophages, fibroblast infiltration, and neovascularization in the new tissue. Granulation tissue may be a precursor to fibrous capsule formation and is separated from the implanted biomaterial device by the cellular components of the foreign body reaction (consisting of macrophages and foreign body giant cells or FBGCs).

Based on this, we examined stages of tissue response using grading scale of capsule quantity and quality around membranes. The four stages of tissue response were initial phase or early tissue repair (7 days), intermediate phase or proliferative stage (14 and 21 days), and late phase or maturation of fibrous capsule (28 days). The higher scores in initial and intermediate phase indicated the lag in healing process, while in late phase the higher scores indicated the speedy maturation of the fibrous capsule.

The amount of fibroblast in DFDBCBM group was relatively lower than the BPCM group after the implantation of biomaterials. This phenomenon might be caused by slightly extended inflammatory response in DFDBCBM group in the immune response evaluation above in which inflammatory cells infiltration in DFFDBCBM group was relatively higher than BPCM group at the end of

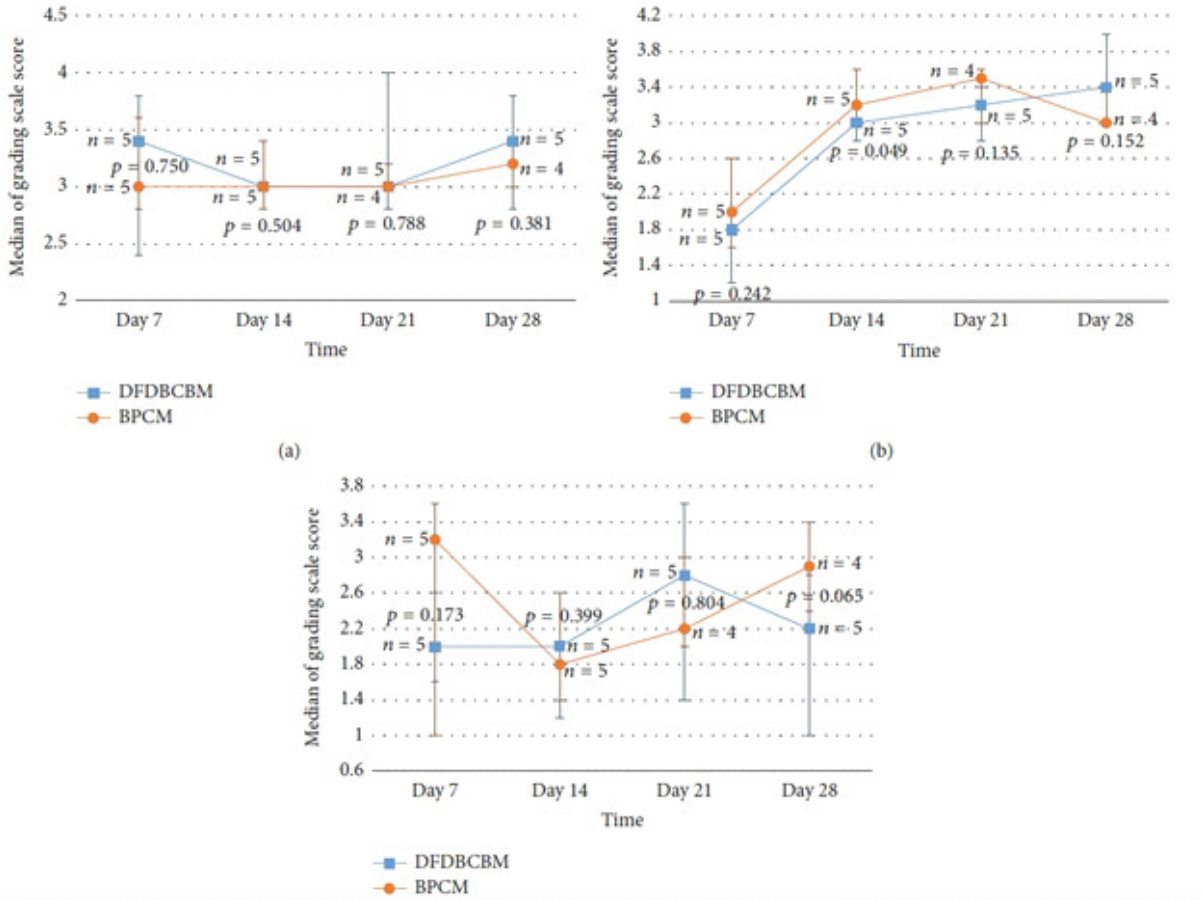

Fig. 2. Analysis of tissue response following subcutaneous implantation of BPCM and DFDBCBM. There was no significant difference in capsule quantity (a), capsule quality (b), and interface quality (c) between the two groups throughout the observed healing periods.

day 7 after implantation although it was not statistically significant

The quality of fibrous capsule analysis showed that both BPCM and DFDBCBM groups exhibited constant increase in capsule quality without any significance difference between the two groups along the observation period. This indicated that normal fibrous encapsulation, along with normal capsule maturation, had occurred in both groups without any signs of prolonged inflammations.

The formation of foreign body giant cell is the initial response of biomaterial implantation that occur in the interface (arean between biomaterial and the surrounding tissue). *Foreign body reaction*is important in the first and second weeks post implantation. It determine the biocompatibility and a favorable outcome. *Foreign body response*indicate a non specific immune reaction as a result of implantation of any foreign material. This showed by infiltration of any inflammation cells and regeneration of the damage tissues. The response of tissues to a foreign material was much the same as the standard response to tissue injury; however, inflammation and macrophage activation did not resolve at the later stages and persistence of inflammatory cells, in particular macrophages, occurred (Sheikh *et al.*, 2015). Macrophages had been shown to respond and naturally bound to almost all biomaterials once implanted, including collagen (Calle *et al.*, 2006). It had been demonstrated that macrophages participate in the degradation of biomaterials by the release of a variety of enzymes (Kang *et al.*, 2016), mediators of degradation such as reactive oxygen intermediates (ROIs), enzymes, and acid between the cell membrane and biomaterial surface (Ross *et al.*, 2002). Macrophages could fuse and became foreign body giant cells (FBGCs), which were observed at biomaterial-tissue interface of implanted devices and tissue engineering scaffolds (Brodbeck *et al.*,

2005). It was suggested that implant sites that had a greater number of macrophages and foreign body giant cells had more fibrosis and encapsulation of the biomaterials (Mitragotri, 2009). This phenomenon was evident in the result of this study in which macrophages were seen to populate the surface of the membranes, inside porosities of BPCM structures and at DFDBCBM cleavage areas which confirmed the role of macrophages in membrane biodegradation.

The results also showed that no statistical difference was found regarding the interface tissue quality in both types of membrane, which logically meant that there was no excessive number of macrophages and FBGC in the interface tissue of DFDBCBM. This could be attributable to the facts that both types of membrane were composed of bovine fibrillar collagen type-I although they were taken from different parts of the body.

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