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by Pratiwi Soesilowati

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INFLAMMATORY RESPONSE IN RAT'S DORSUM AFTER SUBCUTANEOUS IMPLANTATION OF DEMINERALIZED FREEZE DRIED BOVINE CORTICAL BONE MEMBRANE

LULUK YULIANANI[1], DAVID B. KAMADJAJA[2,4,*], NURUL MAULIDAH[1] AND PRATIWI SOESILOWATI[3]

[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

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Abstract– Guided bone regeneration (GBR) is a surgical procedure that uses barrier membranes with or without particulate bone grafts. It is used to enhance bone growth and commonly uses collagen membrane made from bovine pericardium (BPCM). However, it has been associated with prolonged biodegradation. Therefore, development of Demineralized Freeze Dried Bovine Cortical Bone Membrane (DFDBCBM) as an alternative collagen membrane that can be used as GBR is needed. The cellularity inflammation response of DFDBCBM yet needs to be analyzed to reveal its immunogenicity. This study aims to determinate immunogenicity of DFDBCBM compared with BPCM when implanted subcutaneously. We used 30 rats as samples which were divided into 2 groups, each consist of 15 samples. Dorsum of rats were subcutaneously implanted with DFDBCBM in the first group; and the second group was implanted with BPCM. Samples were sacrificed after 2, 5, and 7 days for histology examination using Hematoxillin Eosin (HE) staining. Number of polymorphonuclear (PMN), lymphocyte and macrophages were observed under light microscope. The data was then analyzed statistically at the significance value of < 0.05. The number of PMN was significantly higher in DFDBCBM than BPCM group in day 2 and 7 after implantation. The number of lymphocyte and macrophages was not significantly different in DFDBCBM than BPCM in day 2, 5 and 7 after implantation. Both groups showed declining pattern along the observation period. Demineralized freeze dried bovine cortical bone membrane does not induce excessive cellular inflammation response, thus it is not immunogenic to be used as guided bone regeneration membrane.

INTRODUCTION

Loss of tooth can cause negative impact such as decrease of alveolar bone dimension because of resorption in the socket wall. To resolve the problem, clinician can apply alveolar bone grafting procedure to stimulate healing and stabilize the dimension of alveolar bone (Lekovic, 2007). The alveolar bone grafting procedure to augment alveolar bone needs GBR method, which is a combination of bone graft material and a membrane placed between bone graft material and soft tissue. The membrane function as protection for bone healing process against fibroblast cell infiltration which tends to induce fibrotic healing in the defect (Buser *et al*., 1993).

Pericardium membrane have shown effective association compared to collagen membrane. They have shown prolonged absorption with real collagen feature. Some in vitro and in vivo studies have been done to evaluate the effectiveness of decellular pericardium membrane to improve bone growth (Bai, 2014).

The use of freeze dried bovine bone graft (FDBX)

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

is a prompt necessity in bone defect reconstruction since the limited human bone donor. Research in DFDBCBM is an expansion from FDBX research oral and maxillofacial surgery especially the GBR method. Since DFDBCBM is a xenomaterial, series of researches are needed to prove DFDBCBM safety and effectiveness in human. Safe is defined as biocompatible trait of the membrane, where there is normal inflammation process, non-cytotoxic, and non-immunogenic to the host tissue. While effective is defined as ability of the membrane to persist (not being degraded) inside host tissue for nothing less than 3 weeks (Olivera *et al*., 2004).

Thus, the purpose of this study is to determine the in vivo inflammation response of DFDBCBM by analyzing the tissue inflammation response after implantation of subcutaneous DFDBCBM compared to pericardium bovine.

MATERIALS AND METHOD

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.

Thirty male Wistar rats used in this study were randomly divided into 2 groups. In experimental group 5×5mm DFDBCBM were implanted in the rats' dorsal subcutaneous tissue while in control group BPCM (Jason Membrane®, Botiss, Germany) was used for the implantation. Five rats from each group were sacrificed at 2, 5, and 7 days after implantation by over sedating them with ether vapor. The implanted membranes were retrieved by removing the membrane together with their surrounding tissues, fixed in 10% buffered formalin solution. The tissue was then embedded in paraffin block and thin section was made using microtome. The sections were then stained with Hematoxylin & Eosin and investigated with light microscope for histology examination. The number of lymorphonuclear or PMN cells, macrophages, and lymphocytes adjacent to implanted membranes was counted to quantitatively determine the immune response to DFDBCBM and BPCM subcutaneous implantation. The inflammatory cells counting was performed "blind"; that is, the microscopic areas chosen were randomly assigned, done by two different persons, and the slides numbers were randomized to make the counting blinded. Statistical analysis was performed using software package IBM SPSS for Windows version 21. The data evaluation was analyzed using one-way analysis of variance. Statistical significance was determined when the 5] value < 0.05.

RESULTS

Histology examination showed that the characteristic of immune response was somewhat different between the two groups. Infiltration of inflammatory cells was evident at the periphery of DFDBCBM while in BPCM inflammatory cells were found both in the periphery and inside the membrane porosities. Intra membrane cell infiltration was more evident in later days after implantation, whereas in DFDBCBM cleavage of membrane structure was noted at day 7 after implantation (Figure 1).

The result of histology cell counting showed that the amount of PMN cells in DFDBCBM group was significantly higher than BPCM group on days 2 and 7 (5] < 0.05) except for day 5 (5] > 0.05). The histogram exhibited that the amount of PMN showed downward or declining pattern in both groups along the time of examination. The result demonstrated that macrophage count was not statistically different in DFDBCBM group than BPCM group (5] > 0.05) at days 2, 5 and 7 after implantation. The result also revealed that lymphocyte count was not statistically different in DFDBCBM group than BPCM group (5] > 0.05) at days 2, 5 and 7 after implantation (Figure 2).

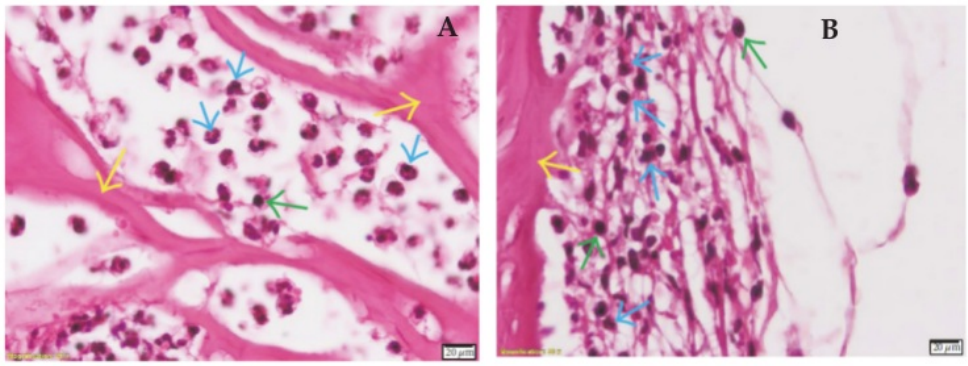

Fig. 1. Microscopic picture of inflammatory cell infiltration at day 2 following subcutaneous implantation of BPCM (A) and DFDBCBM (B). PMN and lymphocyte were the predominant inflammatory cells seen in the tissue surrounding both membranes; blue arrowhead pointing to PMN, green to lymphocytes, and yellow to respective membrane structure (H&E staining, ×1,000 magnification).

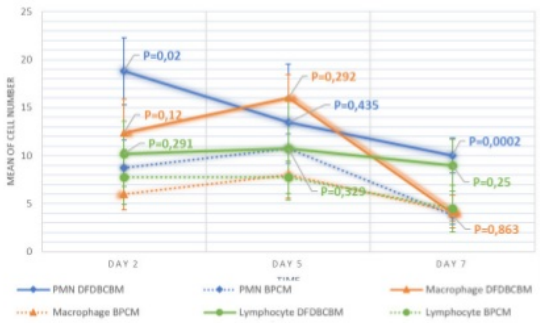

Fig. 2. Distribution of inflammatory cells infiltration following subcutaneous implantation of BPCM and DFDBCBM at 2, 5, and 7 days after implantation (c) Mean of PMN count in DFDBCBM group was significantly higher than BPCM at days 2 and 7. All inflammatory cells infiltration exhibited downward pattern from day 5 to day 7 indicating no prolonged inflammation.

DISCUSSION

Pericardium tissue structure has three layers that mostly formed by collagen tissue and elastic fiber implanted with amorph matrices with porous surface for cellular adherence and proliferation in sufficient density for soft tissue (Rothamel *et al*., 2012). This corresponds to the result of the study where the inflammatory cell spread on the BPCM is up to inside part of membrane, so that the amount of inflammatory cell around the capsule and inside the membrane are quite similar. Whereas in DFDBCBM the amount of inflammatory cell around the capsule are greater than those inside the membrane.

Following the implantation of biomaterials in vivo, host reactions incorporated a combination of many processes including blood-material interactions, provisional matrix formation, inflammation (acute then chronic), development of granulation tissue, foreign body reaction, and fibrous capsule development (Gretzer *et al*., 2006) (Luttikhuizen *et al*., 2006). The provisional matrix was rich in cytokines, growth factors, and chemoattractants that are capable of recruiting cells of the innate immune system to the injury site. The degree of these responses was dependent on the extent of injury during the implantation procedure. The presence of neutrophils (PMNs) characterized the acute inflammatory response (Anderson *et al*., 2008), PMN function as defense agains microorganism invasion especially bacteria (Eroschenko, 2002). The higher PMN infiltration in DFDBCBM group compared to BPCM group observed during the first week of healing confirmed that the material had evoked inflammatory response. These findings could be attributable to two possible factors. First, it could be associated with residual components of processing agent for DFDBCB membrane. Second, the DFDBCBM might be slightly contaminated which may be caused by improper handling of the package of the membrane during implantation procedure or possibly associated with the sterilization procedure during manufacturing process. However, the downward pattern of PMN cells infiltration in DFDBCBM and BPCM group might indicate that inflammatory response decreased with time in both groups which

was important for tissue integration. The result of macrophage and lymphocytes counting which was higher in DFDBCBM group in early post implantation periods again showed that the material had evoked inflammatory response. However, no statistical differences were found between the two groups further down the healing phase. This suggested that both membranes did not cause either excessive or prolonged immune response. Biocompatible implanted materials usually demonstrated early resolution of chronic inflammatory response being no longer than two weeks and being confined to implantation site (Anderson *et al*., 2008). This was important for the membranes to be able to have tissue integration and hence avoid early membrane degradation in later period.

In this study, we do no find prolongation in time of inflammatory response of DFDBCBM. Therefore, the risk of infection is minimal according to the pattern shown in the graph. It can be concluded that DFDBCBM has some potential for application as guided bone regeneration membrane in alveolar bone grafting.

CONCLUSION

Demineralized freeze dried bovine cortical bone membrane does not induce excessive cellular inflammation response, thus it is not immunogenic to be used as guided bone regeneration membrane.

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