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Submission date: 16-Feb-2021 04:40PM (UTC+0800)
Submission ID: 1510631850
File name: fect_via_demineralized_dentin_material_membrane_implantation.pdf (640.66K)
Word count: 4190
Character count: 23530

ISSN 0975-2366
DOI: https://doi.org/10.31838/ijpr/2021.13.01.537

Research Article

Regulating The Fibroblast Growth Into Critical Size Mandibular Bone Defect via Demineralized Dentin Material Membrane Implantation

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Received: 22.10.20, Revised: 21.11.20, Accepted: 20.12.20

ABSTRACT

Mandibular bone damage due to trauma, pathological conditions, and congenital disease create large bone defect or critical size defect that will not heal for life without intervention or healing with forming fibrous encapsulation which is undesirable. Therefore, a Guided Bone Regeneration (GBR) procedure can be performed. Human dentin and bone, as well as bovine dentin, are known to have a very high chemical composition. The researchers considered bovine dentin as an alternative GBR membrane material, namely Demineralized Dentin Material Membrane (DDMM). This research aims to measure the amount of fibroblast after DDMM implantation on a mandibular bone defect. Twenty four rats used as samples. Mandibular bone defect 5x5 mm was made. In the control group defect were not applied membrane, and in the experimental group, the defect was applied DDMM. Six samples were sacrificed on 7, and 14 days post-operation for histology examination using Hematoxylin Eosin (HE) staining. The amount of fibroblast was counted and statistically analyzed using Independent T-test with significance being $p<0.05$. There were no significant differences in the amount of fibroblast after implantation on day 7, and there were significant differences in the amount of fibroblast after implantation on day 14. This result suggests that DDMM implantation is promising to decrease the number of fibroblasts in bone defects and potentially increase bone regeneration.

Keywords: GBR, DDMM, bone, critical size defect, healing

INTRODUCTION

Mandibular bone is the most important dynamic component of the oral and craniofacial region because it is part of the temporomandibular joint (TMJ) and as the attachment site for muscles, ligaments, and salivary glands (Çelik, 2018). Mandibular bone damage due to trauma, pathological conditions (periodontal disease, cysts, tumors, osteomyelitis, and infections), and congenital disease create large bone defect (critical size defect) (Ebrahimi, 2017; Shah et al., 2016). The bone quality and quantity is essential implants or prosthodontic treatment to improve aesthetics and functionality (Ebrahimi, 2017; Mahyudin, 2018). In small defects, the bone can heal without intervention, but if there is a large bone defect, there are not enough bone-forming cells to compensate bone resorption so the defect will not heal for life without intervention. Therefore, a guided bone regeneration (GBR) procedure can be performed (Kamadjaja et al., 2017; Subagio et al., 2018)

GBR is a procedure for bioresorbable or non-resorbable barrier membranes implantation indicated for large bone defects related to aesthetic or functional requirements. In order to improve bone regeneration, it was essential to keep the defect separated from the fibrous organization by inserting membranes following the principle of GBR. The characteristics of the GBR membrane include biocompatibility, cell-occlusiveness, space-making, tissue integration, and clinical management (Dimitriou et al., 2012; Kamadjaja et al., 2017). This barrier membrane has several functions. It protects the healing process of bone defects, increases osteogenesis by preventing the migration of non-osteogenic cells (epithelial cells and connective tissue), maintains the balance of blood clot and bone-forming cells, accumulates growth factors,

stabilizes bone graft, and maintains space for bone growth (Liu & Kerns, 2014; Urban & Monje, 2019).

Human dentin and bone as well as bovine dentin, are known to have a very high chemical composition (70% hydroxyapatite, 20% organic matrix, primarily type 1 collagen, and 10% water). Dentin is a mineralized connective tissue with an organic collagen matrix. It contains bone morphogenetic proteins (BMP) and growth factors such as transforming growth factor-beta 1 (TGF-β1), fibroblast growth factor -2 (FGF-2), platelet-derived growth factor. (PDGF), insulin-like growth factor-1 (IGF-1), and IGF-2, although the amount is lower than bone (Gao et al., 2019; Putri et al., 2020; Sari et al., 2018; I. W. Um, 2017). Based on this situation, the researchers considered bovine dentin as an alternative material in bone regeneration, namely Demineralized Dentin Material Membrane (DDMM).

The healing process of soft tissue and bone consists hemostasis phase which is characterized by the formation of blood clots and presence of platelets and leukocytes, inflammation phase by the action of macrophages and lymphocytes, proliferation phase by the action of fibroblasts and osteoblasts, and remodelling phase. Mandibular bone defects healing through intramembranous ossification lasts for three months, while in long bones healing through endochondral ossification for seven months because the mandibular bone has better vascularization and mechanical environment which makes it is more fixed (Dimitriou et al., 2012; Soesilawati et al., 2019; Yuliati et al., 2020). Fibroblast is a small component of connective tissue found in bone and as the leading producer of type 3 collagen. At the same time, osteoblast is the leading producer of type 1 collagen, which is found in 95% of the 80% protein bone composition (Alberts et al., 2002; Kamadjaja et al., 2017; Sandberg & Aspenberg, 2016). The bone defect healing process can result in the formation of fibrous tissue due to invasion of fibroblasts into the defect space because the migration rate of fibroblast is higher than osteoblast. The formation of mature fibrous tissue leads to undesirable situations such as non-union encapsulation. In bone healing, the concept of GBR membrane implantation can prevent these problems (Aslan et al., 2004).

In this in-vivo study, we aim to determine the effect of DDMM membrane implantation on mandibular bone defects to prevent fibroblasts from connective tissue entering the critical defect bone area on days 7 and 14.

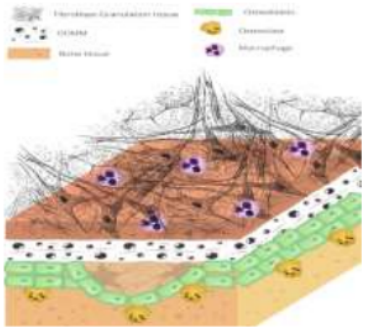

Fig.1: The illustration of GBR procedure using DDMM

MATERIALS AND METHODS

36 Wistar rat *(Rattus norvegicus)* with inclusion criteria, male gender, age 2-3 months, body weight 250-300 g, and healthy condition without any physical disabilities adapted in cages for 7 days to adjust to the new environment. A right environment was made by preventing fear, stress, discomfort, pain, and contracting diseases that could affect the results of the study. The cage is in a room with sufficient air circulation, the right amount of sunlight, away from the noise, and in a dry place so that it does not become a den of disease. In this study, the experimental protocols were approved by the Health Research Ethical Clearance Commission (Universitas Airlangga Faculty of Dental Medicine Number 339/HRECC.FODM/VII/2020).

The experimental animals were divided into two groups consisting of a control group and a DDMM group, each consisting of 18 rats. Rats were anaesthetized with ketamine HCl 20 mg/kg body weight intramuscularly in the femoral region. The hair on mandible region is shaved and disinfected with povidone-iodine 10%. The 5x5 mm defect was made in the mandible using a micro motor, low-speed handpiece, and wheel bur. Irrigation with NaCl 0.9% was used during this procedure. In the control group, the defect was not given a GBR membrane.

In contrast, in the treatment group, the defect was implanted with DDMM size 1x1 cm, which had been immersed in physiological fluids for several minutes. The wound area was sutured using silk thread 3.0. On 7 and 14 days, post-operation, six animals in each group were euthanized using ether to extract their mandibular bone. Tissue samples were fixed in formalin solution, then decalcified in 10% EDTA solution for six weeks. Tissue samples were processed and embedded in a paraffin block, then cut to a thickness of 3 μm and placed in a glass object for Hematoxylin Eosin (HE) staining. Each slide was counted the

number of fibroblasts using a microscope with a magnification of 400x and nine fields of view. On histology, the nucleus of fibroblast cells appears oval, and the cytoplasm is homogeneous and basophilic.

The result was analyzed using a normality test. As data were normally distributed (p>0,05), an Independent T-Test was used to compare the amount of fibroblast in control and DDMM group. The amount of fibroblast shows significantly different if p<0.05.

RESULTS AND DISCUSSION

Fibroblast histomorphometry was performed in both sample groups. The amount of fibroblast in control and DDMM group on 7 days post-operation were not significantly different (p = 0.126), but on 14 days post-operation were significantly different (p = 0.000). The histomorphometry data is shown in table 1.

Table 1: Amount of fibroblast on 7,14, and 21 days post-operation

Fibroblast	Mean ± SD		P values
	Control	DDMM	
Day 7th	45 ± 6,36	35,17 ± 12,96	.126
Day 14th	52 ± 4,56	27,16 ± 1,6	.000

Bone defect undergoes healing through hemostasis stage, which is played by leukocytes and platelets and indicated by the formation of blood clots in the first 24 hours. In the GBR membrane implantation, injured blood vessels cause protein adsorption onto the membrane surface for a few seconds to form fibrin-rich clots and for 60 minutes there is the adhesion of monocytes, macrophages, and platelets to the membrane surface. On day 1-5, blood vessels adjacent to the membrane surface release pro-inflammatory cytokines. Moreover, it chemokines to stimulate the recruitment of inflammatory cells and osteoprogenitor cells to the membrane surface. Neutrophils play an essential role during early phase inflammation; monocytes and lymphocytes play an essential role during late-phase inflammation. Monocytes differentiate into macrophages (M1 and M2) and multinucleated giant cells. M1 releases pro-inflammatory cytokines (IL-6 and IFN-γ), whereas M2 releases anti-inflammatory cytokines (IL-10 and TGF-β) for tissue regeneration. The increase in excess M1 activity causes forming fibrous encapsulation. The fibrous tissue will fill the bone defect and separate the membrane from the host-bone tissue, failing bone defect healing. PMN, macrophages and monocytes also secrete IL-1, IL-6, TNF-α, MCP-1 and SDF-1 to stimulate recruitment of other inflammatory cells, fibroblasts and MSC. Fibroblasts and endothelial cells release VEGF to induce angiogenesis, migration and proliferation of MSCs and osteoblasts. BMPs released during early phase inflammation also play an essential role in the proliferation and differentiation of MSCs into fibroblasts and osteoblasts. Fibroblasts secrete collagen to form granulation tissue. Recruitment of cells for bone tissue repair lasts 5-15 days. At week 3-4, granulation tissue and fibrous encapsulation are formed around the membrane area (Haque et al., 2020; He et al., 2020; Sheikh et al., 2015).

In the control group, the amount of fibroblasts increase from 7-14 days post-operation, and it was higher than the DDMM group. Day 7 is the early phase of proliferation phase so the differentiation of MSCs into fibroblasts and osteoblasts is induced by pro-inflammatory cytokines (PDGF, TGF-β and BMP-2) released by neutrophils and lymphocytes in the inflammatory phase. According to Aslan et al., on the 10th-day post-operation, a posterior tibial bone defect that was implanted with resorbable GBR membrane showed active trabecular bone formation (spongiosa). In contrast, defects that were not implanted with the GBR membrane were filled with fibrous tissue (Aslan et al., 2004). The increase number of fibroblasts until the 14th day occurs because defect that is not provided with a barrier in the form of a GBR membrane causes the entry of non-osteogenic cells (epithelium and fibroblasts) into the defect area (Liu & Kerns, 2014; Urban & Monje, 2019). Based on in vivo GBR study conducted by Dahlin et al. in 1988, at the 6th week of maxillofacial defects that were not given GBR membrane found fibrous tissue non-union due to invasion of connective tissue and or muscle tissue. In the inflammatory phase, there is a release of cytokines and growth factors PDGF, TGF-β, and BMPs which are known to be responsible for migration, recruitment, proliferation and differentiation of MSCs to form new bone tissue. Fibroblasts with a higher migration rate than osteoblast cells invade the defect (Aslan et al., 2004).

The number of fibroblast in the mandibular bone defect at day 7 and 14 post-operation in both groups can be seen in Fig 2.

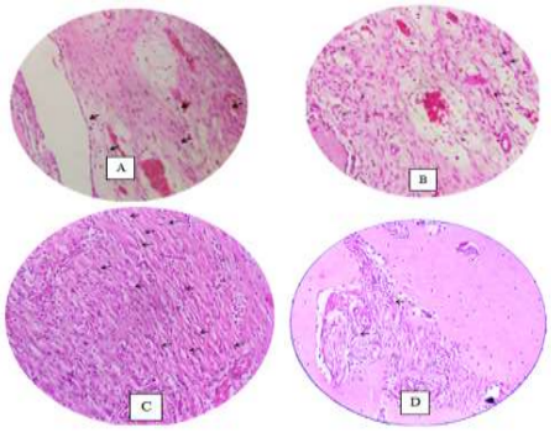

Fig.2: Fibroblast (black arrow) in the mandibular bone defect with HE staining and counted using a microscope (400x) (A) Day 7 post-operation in the control group. (B) Day 7 post-operation in DDMM group. (C) Day 14 post-operation in the control group. (D) Day 14 post-operation in DDMM group.

The amount of fibroblasts decreased from 7-14 days in the DDMM group.. The GBR membrane was created to prevent soft tissue cell invasion, but soft-tissue growth still occurred up to 1 mm below the membrane (Dimitriou et al., 2012). According to the research conducted by Bubalo et al., who gave GBR membranes with different thicknesses, fibroblasts remained until six months after implantation. It is not known whether the soft tissue that grows under the membrane will mineralize for a long time or whether fibrous tissue will form (Bubalo et al., 2012). Fibroblast can differentiate into osteoblast-like cell through direct reprogramming using factor transduction Runt-related transcription factor 2 (Runx2), Osterix, and Octamer-binding transcription factor (Oct) 3/4, L-Myc (RXOL) so fibroblast can produce calcified bone matrix and expresse osteoblast spesific marker (Chang et al., 2019; Yamamoto et al., 2015). Reprogramming human fibroblast cells through induced pluripotent stem cells (iPSC) can also lead to osteogenic differentiation of fibroblast cells into osteoblast-like cells, but consideration is needed in the use of iPSCs such as specialized technical resources for direct reprogramming and its consequences such as teratomas in regenerative applications (Clarkin et al., 2020).

The dentin microstructure contains collagen fibers and growth factors such as IGF-1, IGF-2, BMPs (BMP-2, BMP-4, BMP-7), TGF-β, VEGF, PDGF, and FGF-2 (Sari et al., 2018; I.-W. Um et al., 2017). BMP-2 induces osteoblast differentiation from MSC and BMP-7 promotes angiogenesis directly. FGF is known not only to play an important role in angiogenesis but also to provide mitogenic effects of MSC. In bone fractures, VEGF is released from the blood clot and promotes endothelial cell development to induce vascular invasion. In in vivo studies, VEGF plays a role in bone maturation but does not increase the amount of bone formation (Turri et al., 2016; Wang & Yeung, 2017). In bone healing, FGF-2 is known to stimulate endothelial cell migration and proliferation, fibroblasts and osteoblasts. FGF-2 and BMP-2, together with the collagen structure in the membrane, are important factors for the migration, proliferation and differentiation of different cells in the membrane (Turri et al., 2016).

The GBR membrane implantation procedure causes different immune responses during bone regeneration. It is so because the membrane is considered as a foreign body and the immune response depends on physical membrane characteristics (stiffness, porosity, roughness, hydrophobicity, surface charge, and membrane geometry) and the chemical content. In a rigid membrane, the neutrophils migration rate are slower, but the more remarkable directional persistence. Macrophages migrate more rapidly when the membrane is implanted. Increased secretion of IL-10 and decreased secretion of IL-12 and TNF-α occurred in thicker membranes (He et al., 2020). DDMM has 300 μm thickness which decreases the formation of fibrous encapsulation tissue.

The efficiency of the GBR membrane to regenerate bone is based on the characteristics of the membrane, which include composition, thickness, porosity, perforation size, and local pH (Dimitriou et al., 2012). pH is related to the rate

of membrane biodegradation which can affect the space making ability. The thickness and stiffness of the membrane are related to the formation of space for bone regeneration. The pore size of DDMM less than 5μm with a membrane thickness of 300 μm is able to withstand the entry of fibroblasts whose size is 10-20μmand optimal for neovascularization (Iordache, 2019; Pradhitta, 2019). Microporous membranes (<10μm) showed better bone regeneration and cell attachment (Elgali et al., 2017; Herda & Puspitasari, 2016).

CONCLUSIONS

DDMM membrane implantation can prevent fibroblast growth into bone defect characterized by a decrease in the number of fibroblasts in bone defects and potentially increase bone regeneration. These results prove that DDMM implantation in critical size bone defects is a promising treatment option for osteogenesis.

Conflict of Interest: The authors declare that there is no potential conflicts of interest with respect to the authorship and/or publiaction of this article.

Acknowledgements: The study was financially supported by the Ministry of Research, Technology and Higher Education, the Republic of Indonesia, Grant no. 616/UN3.14/PT/2020.

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