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Article

Demineralized Dentine Material Membrane As Barrier Membrane for Bone Regeneration

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Abstract: Mandibular critical size defect (CSD) due to pathological conditions, trauma, and congenital disease can not heal spontaneously and predominantly filled with fibrous tissue. Therefore, a Guided Bone Regeneration (GBR) combined with bone grafting can be performed. The researchers considered using Demineralized Dentin Material Membrane (DDMM) from bovine dentine as an alternative GBR. This study aimed to determine the amount of fibroblast and collagen density after DDMM and bone graft implantation on CSD. Thirty-six *Rattus norvegicus* rats were used as samples. Mandibular bone defect 5x5 mm was made, then filled with bone graft and covered with Bovine Pericardium Collagen Membrane (BPCM) in the control group and DDMM in the treatment group. Six samples were sacrificed on 7, 14, and 21 days post-surgical for histology examination. There were no significant differences in the amount of fibroblast and collagen density (p-value > 0,05). The amount of fibroblast is lower and the collagen density is higher in treatment group. DDMM has microporosity to prevent connective tissue ingrowth and dentine tubules to allow growth factors release. DDMM and bone graft implantation can reduce the amount of fibroblasts and increase collagen density of CSD which potentially being used as a CSD alternative treatment for bone regeneration.

Keywords: GBR, bone graft, DDMM, fibroblast, collagen fiber.

1. Introduction

Mandibular bone damage due to pathological conditions (cysts, tumors, osteomyelitis, periodontal disease, and infections), trauma, and congenital disease create large bone defect or critical size defect (CSD) [1]. The reconstruction of CSD is a significant problem for Oral and Maxillofacial Surgeons because it will not heal spontaneously without intervention. It can regenerate>10 % of the lost bone during the patient's lifetime [2]. CSD heals predominantly with fibrous tissue, not bone because there are not enough bone-forming cells for bone regeneration. Therefore a guided bone regeneration (GBR) procedure can be performed [3,4].

Guided Bone Regeneration (GBR) is a mechanical barrier membrane placement to protect blood clots and isolate the bone defect from the surrounding connective tissue, thus providing bone-forming cells for bone regeneration [5]. There is the non-resorbable and resorbable membrane. The non-resorbable GBR membrane such as polytetrafluoroethylene (PTFE), expanded polytetrafluoroethylene (e-PTFE), titanium (Ti), and titanium-reinforced PTFE (Ti-PTFE) have been widely used and are commercial. Based on the material, the resorbable GBR membrane can be derived from synthetic materials such as Poly-Lactic Acid (PLA) and Poly-Glycolic Acid (PGA) natural materials such as bovine atau porcine [6]. In resorbable membranes, which tend to be less rigid, the combination of bone graft administration can prevent the membrane from collapsing and maintaining defect space [7,8].

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Bone graft material can be derived from humans (autogenous, isograft, and allograft), other species such as bovine, porcine, equine, coralline, and algae (xenograft), or synthetic materials (alloplastic) [9]. The type of bone graft widely used in bone reconstruction in orthopedics and oral surgery is hydroxyapatite (HA). It is the main bone component (55-56%) and teeth, containing growth factors anti-infective. The microstructure resembles human bone (biomimetic). Bone regeneration in GBR and bone grafting procedures can be achieved through different mechanisms, including osteoinduction, osteoconduction, and osteogenesis [9–11].

Dentin is a mineralized connective tissue with an organic collagen matrix that contains bone morphogenetic proteins (BMP), platelet-derived growth factor (PDGF), fibroblast growth factor -2 (FGF-2), transforming growth factor-beta 1 (TGF-β1), and insulin-like growth factor-1 (IGF-1), and IGF-2. However, the amount is lower than bone. Human bone, human dentine, and bovine dentin have similar chemical compositions containing 70% hydroxyapatite, 20% organic matrix, and 10% water [12–15]. Based on the study, the researchers considered bovine dentin as an alternative material in bone regeneration, namely Demineralized Dentin Material Membrane (DDMM), which acts as osteoconduction and osteoinduction.

Collagen is a composition of the organic matrix of bones. In bone, collagen is synthesized by osteoblasts, whereas in connective tissue, collagen is synthesized by fibroblasts [16]. Bone has 95% collagen type 1 from 80% protein, which plays a role in maintaining the extracellular matrix's stability for mineral deposition and binding with other macromolecules. Collagen type 1 has a "hole zone" that can promote calcium HA crystals' deposition between the collagen fibers to provide bone strength and density [17–19]. The bundle of irregular collagen fibers and osteocytes forms woven bone, which is then replaced in the remodeling phase by lamellar bone with better mechanical strength [20]. The bone defect healing can result in fibrous tissue formation due to the invasion of fibroblasts into the defect area because the migration rate of fibroblasts 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 [21].

The purpose of this research was to determine the amount of fibroblast and collagen fiber density after DDMM and bone graft implantation on CSD on days 7, 14, and 21 post-implantation procedure.

2. Materials and Methods

This research is Post Test Only Control Group Design. The observation of fibroblast and collagen fiber density in rat's mandibular critical bone defect histologically after implantation of BPCM and bone graft (control group) and DDMM and bone graft (treatment group) on day 7, 14, and 21. BPCM using Jason membrane, a commercial product, and DDMM processing was performed at Tissue Bank/Center for Biomaterial and Stem Cell, Dr. Soetomo General Hospital, Surabaya.

Each group contains 18 Wistar rats *(Rattus norvegicus)* age 2-3 months, bodyweight 250-300 g, and healthy condition adapted in cages for seven days to adjust to the new environment. The rats were anesthetized with ketamine HCl 20 mg/kg body weight intramuscularly in the femoral region. The 5x5 mm defect was made in the mandible using wheel bur and irrigated with NaCl 0.9% during defect making. The defect was filled with HA bone graft, then covered with GBR membrane, immersed in physiological fluids for several minutes. The wound area was sutured using silk thread 3.0. On 7, 14, and 21 days post-operation, six animals in each group were euthanized. Tissue samples were fixed in formaline 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 and collagen density using a microscope with a magnification of 400x. The histopathology (HPA) of fibroblasts is the nucleus appears oval, and the cyto-

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plasm is homogeneous and basophilic. Meanwhile, collagen fibers stained pink [21]. 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.

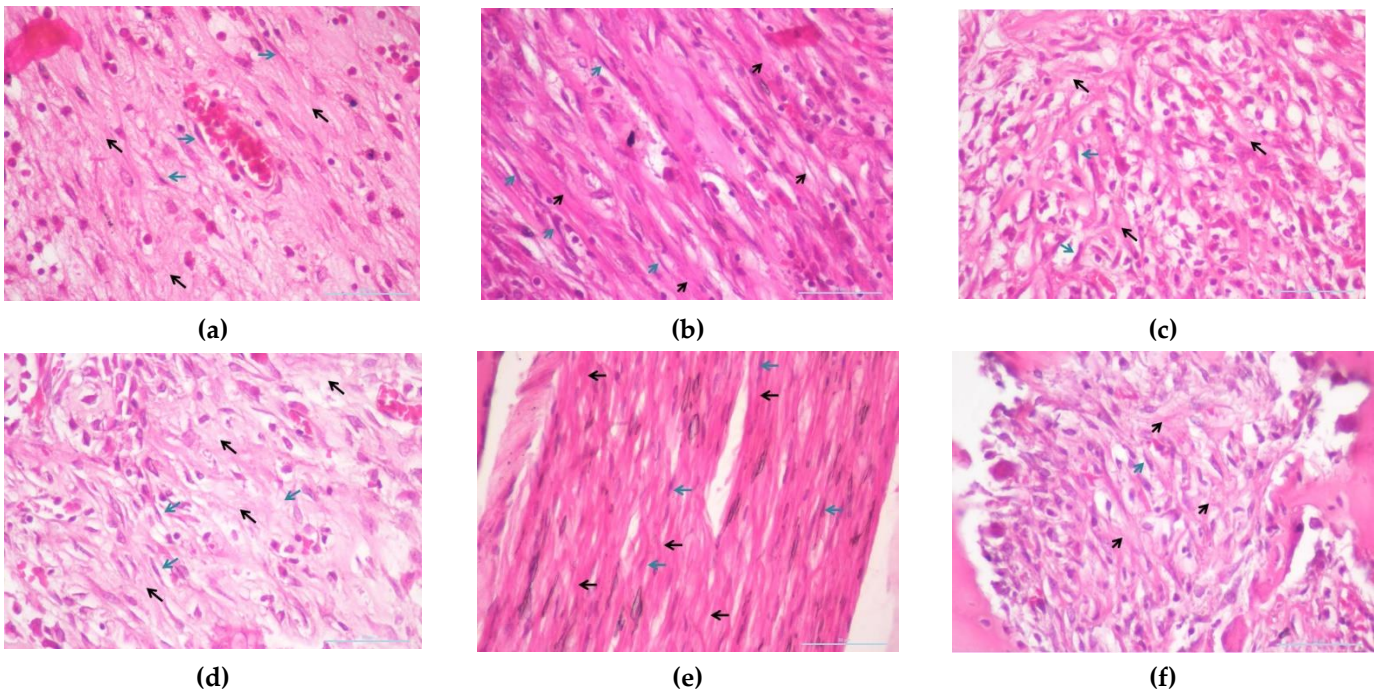
The result was analyzed using a normality test. As data were normally distributed (p>0,05), an Independent T-Test was used to compare the amount of fibroblast and collagen density in the control and treatment groups. The amount of fibroblast and collagen fiber density shows significantly different if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

3. Results

Fibroblast and collagen density histomorphometry was performed in both sample groups. The amount of fibroblast and collagen density in the control and treatment group on 7, 14, and 21 days post-operation were not significantly different (p>0,05). The histomorphometry data is shown in Table 1, and the histopathology feature is shown in Figure 1.

Table 1. The amount of fibroblast and collagen fiber density on 7, 14, and 21 days post-implantation.

Variable	Day	Mean ± SD		*P-value*
		Control group (BPCM+bone graft)	Treatment group (DDMM+ bone graft)	
Fibroblast	7th	30 ± 2,85	29 ± 6,36	.820
	14th	26 ± 4,92	25 ± 3,16	.734
	21st	22 ± 1,6	18 ± 6,49	.250
Collagen fiber	7th	7 ± 1,33	8 ± 2,61	.501
	14th	13 ± 1,89	15 ± 1,89	.098
	21st	10 ± 2,22	10 ± 3,34	1.000



(a) **(b)** **(c)**

(d) **(e)** **(f)**

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Figure 2. Histopathology (HPA) of fibroblast (blue arrow) and collagen fiber (black arrow) on 7, 14, and 21 days (left to right) in mandibular bone defect post-implantation of bone graft combine with **(a)** ,**(b)**, **(c)** BPCM and **(d)** ,**(e)**, **(f)** DDMM (HE; 400x).

4. Discussion

Bone defects undergo healing through four phases there are hemostasis, inflammation, proliferation, and remodeling. In the first 24 hours after the defect is formed, thrombin was released, caused by the blood vessel's rupture under the periosteum. It stimulates the formation of blood clots by platelets. Tissue hypoxia caused by blood vessel damage promote platelet to release IL-1, IL-6, TNF- α, PDGF and TGF-β to recruit neutrophil and monocyte [22]. IL-1 and IL-6 peak 24 hours post-injury and starts undetectable 72 hours post-injury. In the early inflammatory phase, neutrophil differentiates into PMN to remove pathogens, tissue debris, and thrombus. Neutrophil also release IL-1, TNF-α, and MIP-1 to monocyte differentiation into macrophage [22]. Macrophages will phagocyte necrotic cells at the bone ends, bacteria, and secrete inflammatory cytokines and growth factors such as BMP-2, BMP-5, BMP-7, b-FGF, TGF-β, PDGF, and IGF, which are responsible for migration, recruitment, proliferation, and differentiation of MSC into angioblast, chondroblasts, fibroblasts, and osteoblasts [23]. VEGF, PDGF, and FGF as angiogenic factors secreted by macrophages can also stimulate MSC differentiation into endothelial cells for angiogenesis [24].

On the 7th day post-injury, the early phase of the proliferation phase is characterized by the differentiation of MSCs into fibroblasts and osteoblasts. The inflammatory cells will secrete IL-1β, PDGF, and FGF to stimulate fibroblast proliferation, while fibroblast migration to the wound area is stimulated by TGF-β [25,26]. Implantation of osteoconductive biomaterials can be used as a scaffold or medium to create a suitable atmosphere for adhesion, proliferation, and differentiation of MSCs to osteoblasts as collagen producers [27]. The peak amount of fibroblast and collagen on day 14th is the critical period of the proliferation phase. Day 21st post-injury reduces fibroblast and collagen density because the remodeling phase has just started. Osteoclasts secrete hydrogen ions and the enzyme lysosome cathepsin K, which degrades all components of the bone matrix, including collagen, to form a Haversian lacuna basin, which will be filled with new bone matrix by osteoblasts in the formation stage in the remodeling phase [28,29]. Collagenase (MMP-8 and MMP-13), produced by osteoclast, also degrades collagen fiber causes decreasing collagen density on the 21st day [30].

Synthetic bone graft (alloplastic) has osteoconductive properties that can scaffold for adhesion and development of osteogenic progenitor cells or MSC and new blood vessels [9,31,32]. The increase in the number of MSCs which proliferate and differentiate into preosteoblasts causes collagen synthesis to increase. In the study of Vidyahayati et al. (2016), bone graft HA is known to increase the number of osteoblasts during bone formation on the 14th day. Bone graft particles with a porosity> 50% of the graft volume and pore sizes of 200-800 show optimal bone development as they allow for osteoblast migration, adhesion, and proliferation [9]. The particle size of the bone graft coralline HA used in this study was estimated to have porosity> 70% and pore size of 500-600 μm so that osteoblast proliferation was good and collagen production increased [34].

The number of fibroblasts and collagen density at days 7, 14 and 21 in both groups showed no significant difference. It can be due to the size of the porosity of DDMM and BPCM not much different. DDMM has a porosity of 3.4 μm, while BPCM has a porosity of 3.9 μm. Microporosity (<1-5 μm) is beneficial in vivo because it can stimulate bone regeneration [35]. Larger pore size will allow soft tissue-forming cells to grow faster, thus inhibiting infiltration and osteogenic cell activity [36]. The higher porosity and larger pore size may be beneficial in vivo because it can stimulate bone regeneration [35]. In the cytotoxicity test (MTT assay), DDMM is known to have the potential to increase the number of MC3T3-E1 osteoblasts, which are producers of collagen in bone [37]. The demineralization process in making DDMM is known to maintain the content of type 1 collagen. BMP-2 released by DDMM increases the differentiation of MSCs into

osteoblasts so that the amount of collagen secreted is increasing [5]. The membrane thickness also affects the space-making ability, affecting the growth of soft tissue to the defect area. The thickness of DDMM was 300 μm and BPCM 150 μm, which made DDMM better to prevent an invasion of fibroblasts into the defect area [35].

5. Conclusions

DDMM and bone graft implantation can reduce the amount of fibroblasts and increase collagen density of CSD which potentially being used as a CSD alternative treatment for bone regeneration.

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