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Effect of Collagen Membrane on Tumor Necrosis Factor-Alpha (TNF-α) Level in Wound Healing Process in Rats

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Abstract: ***This study aimed to measure the TNF-α level in rat blood serum during wound healing process using collagen membrane. Methods: 24 male Wistar rats were divided into two groups: treatment and control. A bone defect was created on the rat's jaw using a 2 mm diamond bur. The defect was then covered with collagen membrane in the treatment group and without collagen membrane cover in control group. Results: the average TNF-α level in the treatment group was 209.7 pg/mL while in the control group the level was 487.3 pg/mL. There was a significant difference in the average TNF-α level between the treatment and control groups (p= 0.03). Conclusions: the use of collagen membrane can reduce the TNF-α level in rat's wound healing process.***

Keywords: collagen membrane, TNF-α, wound healing

1. Introduction

Collagen has been frequently used in pharmacy applications and as biomedical materials. There are various uses of collagen biomedically including for cosmetic surgery, for patients with burns, bone reconstruction, bone defect filler in the form of collagen sponge, hemostatic agent, wound dressing, slow drug delivery system, and as GTR membrane[1],[2]. One of the uses of collagen in dentistry is as the barrier membrane used in the guided tissue regeneration (GTR) or guided bone regeneration (GBR) procedure due to its good biocompatibility and its ability to accelerate wound healing[3].

The technique of GTR and GBR is based on the concept of preventing the apical downgrowth of gingival epithelium inside the wound area. The placement of barrier membrane between flap and root or bone surface can prevent the penetration of epithelial tissue or gingival connecting tissue into the dental root surface [4],[5]. There are two types of barrier membrane used: resorbable and non-resorbable membranes. Recently, collagen barrier membrane has been used widely for periodontal defect regeneration due to its bio-resorbability that makes it possible to avoid second surgery for membrane removal[4]. Among different materials that have been experimentally and clinically examined, collagen is a good option and is considered to meet various requirements for bio-absorbable membrane[4]. The advantage of collagen is attributable to the fact that it is hemostatic, fibroblast chemotactic, easy to manipulate and able to increase tissue thickness [3], [6].

The wound healing process consists of three phases, i.e. inflammation, proliferation, and remodeling phases. The inflammation phase covers vasoconstriction, hemostasis, and inflammation cell infiltration. Angiogenesis, collagen tissue deposition, granulation tissue development, and epithelial cell migration are seen in the proliferation phase. The remodeling phase is marked by tissue and collagen remodeling, as well as wound retraction[7],[8]. Wound healing is a complex process involving interactions of various cells, cytokines, and growth factors[8,9]. Tumor necrosis factor α (TNF-α) is a pro-inflammatory cytokine that plays a role in the immune response towards infections and wounds. TNF-α affects many aspects of wound healing including hemostasis, increased vascular permeability, endothelial proliferation, increased collagen production, and platelet derived growth factor(PDGF) production[9],[10]. The aim of this study was to analyze the effect of collagen membrane on TNF-α level during rat's wound healing process.

2. Methods

This is an *in vivo* study on male Wistar rats, aged 2 – 3 months and weighed 250-300 grams. The source of collagen was white snapper fish scale collagen added by chitosan which was produced by the National Nuclear Energy Agency (*BadanTenaga Atom Nasional*, BATAN). As many as 24 rats were included in this study. The rats were divided into two groups: treatment group that received collagen membrane application and control group that did not receive collagen membrane application. This study was conducted in the Animal Hospital IPB Bogor. The study protocol and animal treatment had been approved by the animal care and use committee of the Faculty of Veterinary Medicine, Bogor Agricultural Institute (*InstitutPertanian Bogor*, IPB).

2.1 Collagen membrane preparation

Collagen was extracted using 0.5 M acetic acid at 4˚C for 3 days with periodic stirring. Wet collagen was dried using a freeze drier to produce dry collagen. The chitosan solution was prepared with 2% chitosan in 1% acetic acid or, in other words, 2 gram chitosan in 100 mL acetic acid to get a gel-like appearance. Collagen addition was performed by adding 1 gram collagen into the chitosan solution and mixed together using a homogenesis tool until it was homogenous. The collagen and chitosan mixture was then poured into a Petri dish until the desired thickness was gained. The mixture was then frozen in the freeze drier. The sterilization process of the membrane includes gamma ray irradiation with 25 kGy dose for 1–3 hours, depending on the amount of materials being sterilized.

2.2 Surgical procedure

The 24 Wistar rats were purchased from a local vendor in Bogor City. Anesthesia was performed using 75 mg/kg BW ketamil and 10 mg/kg BW xylazil, injected intraperitoneally. Incision was performed extraorally using no. 15 blade in buccal regions, followed by blunt dissection up to the angle of mandible. A 2 mm defect was created on the mandibular bone in the area of the angle of mandible using a low speed bur with a diameter of 2 mm along with 0.9% NaCL irrigation. The collagen membrane was placed to cover the bony defect in the treatment group. The incision wound was closed by suturing using a 5.0 nylon thread. Necropsy of 3 rats from each group was conducted on Day 3, 7, 14, and 21 by exsanguination under anesthesia. Around 2cc of Blood was withdrawn intracranially for TNF-α examination using ELISA method.

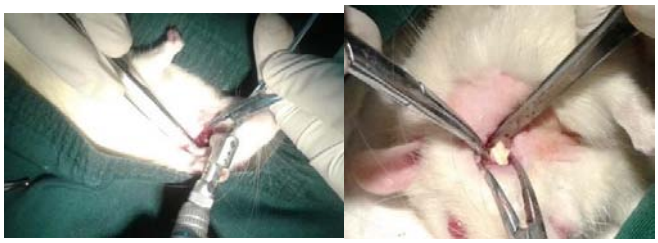

Figure 1: Bone defect creation on rat's jaw using 2 mm round bur. Collagen membrane application covers the bone defect created.

2.3 Enzyme-linked immunosorbent assay (ELISA)

Blood samples were centrifuged with 1500 rpm speed for 15 minutes to get blood serum. The TNF-α level in rat's blood serum was measured by ELISA method using *rat TNF-α Elisa Kit MyBiosource*(Tokyo, Japan). The minimum TNF-α level detected was 15.629 pg/mL and was read using an *ELISA reader* at 450nm wave length.

3. Results

A study on the effect of collagen membrane applications on TNF-α level by comparing it with no collagen membrane application has been performed on 24 subjects that were divided into four measurement times: Day 3, 7, 14 and 21. Three rats were measured in each measurement time. On Day 3 and 7, no TNF-α was detected in the rat's blood. The average serum TNF-α level in the treatment group is listed in table 1. Since the study data was not normally distributed based on the result of normality testing, data transformation was performed to meet data normality by using a transformation logarithm. The complete results of this study are described in table 2 and figure 2. The two-way Anova statistical analysis showed that there was a significant difference in TNF-α level between treatment and control groups (p=0.03).

Table 1: Average TNF-α level (pg/mL) in treatment and control groups

Observation Time (day)	Treatment	
	Treatment group	Control group
Day 3	-	-
Day 7	-	-
Day 14	200.6 (11.90)*	565.6 (475.5)
Day 21	218.8 (74.6)	409 (1.0)
Total	209.7 (51.9)	487.3 (312.7)

Notes: Treatment (collagen membrane)
Control (without collagen membrane)
*Mean (SD)

Table 2: Comparison of mean log (TNF-Alpha) between treatment and Time measurement

TNF-Alpha (log)	Treatment		Total
	Collagen membrane	Without Collagen membrane	
Time measurement : Day of 14	2.3018 (0.0258)*	2.5897 (0.5194)	2.4457 (0.3647)
Day of 21	2.3122 (0.0954)	2.6117 (0.0011)	2.4671 (0.1839)
Total	2.3122 (0.0954)	2.6007. (0.3287)	

Notes: *) Mean (standard deviation)
Comparison between Time measurement : p value = 0.901
Comparison between treatment : p value = 0.033 (One tailed)
Interaction between Time measurement and treatment : p value = 0.997

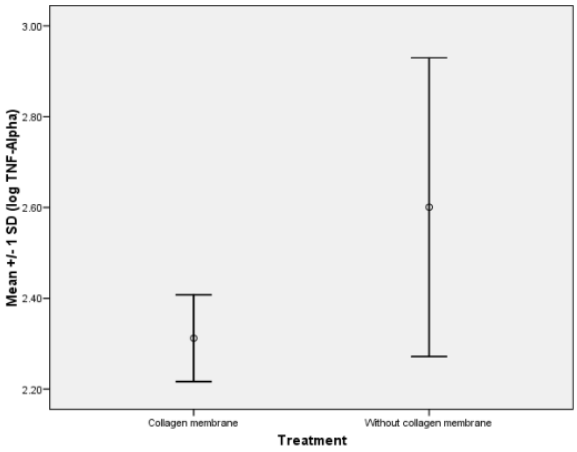

Figure 2: Mean and Standard Deviation of TNF-Alpha (log transformation) between two treatments

4. Discussion

Wound healing is a complex process that involves extracellular matrix component activation, enzyme remodeling, cells, growth factors, and cytokines. The molecular mechanism underlying the healing process is not clear yet and further research is still ongoing [9]. TNF-α is the main cytokine in acute inflammation response and plays an important role in the initial response to wound or bacteria. Excessive TNF-α production is attributed to multisystem organ disorders and increased morbidity and mortality in inflammatory disease, due to its impact on macrophage and neutrophil activation [8],[11]. This cytokine is important for wound healing but excessive production reflects unhealed wound such as in the development of ulcer or inflammatory disease[11]. TNF-α has an important role in the initial inflammation response in wound healing; however, persistent presence of this cytokine locally and systemically may disturb wound maturation [8].

In this study, the group with collagen membrane has an average TNF-α level of 209.7 pg/mL while the group without the membran has a level of 487.3 pg/mL. The average TNF-α level in the group with collagen membrane is lower than that of the control with a difference that is statistically significant $p< 0.05$. This shows that the use of collagen membrane can reduce TNF-α level and that the membrane has the potential to support wound healing process.

Regenerative procedures using the barrier membrane technology have been applied in periodontal therapy and dental implants. Collagen membrane is generally used for the guided tissue regeneration (GTR) and guided bone regeneration (GBR) procedures. The adherence of connective tissue cells to the inside part of the collagen membrane promotes periodontal regeneration and an attachment can help to stabilize the blood clot and integrate the membrane into the tissue[12]. Collagen is used as membrane in GTR or GBR application because it is the main extracellular molecule in periodontal connective tissue, chemotactic for periodontal ligament fibroblast, acts as scaffold for early vascular and tissue growth[12],[13].

Collagen consists of basic molecules such as lysine and arginine, and specific cell attachment site, i.e. arginine-glysine-aspartate (RGD). The RGD actively stimulates cellular attachment by binding with integrin receptor and this interaction plays an important role in cell growth and in the differentiation and overall regulation of cell functions [13],[14]. One of the weaknesses of collagen is that it is rapidly degraded and its tensile strength is low. For this reason, a biogradable polymer is mixed with collagen to produce a scaffold. In this study, collagen that is made of white snapper fish scales was mixed with chitosan for creating membrane and improving the tensile strength. Various studies stated that collagen from white snapper fish scale has quite good physical characteristics to be used as wound dressing materials. The results of SEM analysis show a porous surface that can be used for alternative collagen source for various biomedical materials[15]-[17].

In this study, the TNF-α level on Day 3 and 7 in both membrane treatment and control groups was not detected, which might be due to a concentration not detectable of ELISA kit used (12.625-1000 pg/mL). This result is different with study done by Alberto Turri on TNF-α expression in rat's bone defect when collagen membrane is used. This study suggested that the expression of TNF-α in the membrane defect group increased 2.5 higher than that of the control on Day 3 and reduced significantly on Day 6. After 28 days, there was no difference between the two groups [18]. It was also stated that the expression of TNF-α in the bone defect was analyzed using quantitative polymerase chain reaction (qPCR), while in this present study, the TNF-α level in blood serum was measured using ELISA method.

The effect of the inflammatory cytokine during bone regeneration *in vivo* is still controversial. Pro-inflammatory cytokine level decreases at the end of the inflammation stage and increases again in the remodeling phase around 2 weeks after injury. TNF-α and IL-1β increases on Day 14. The role of TNF-α in the initial recruitment and osteogenic differential of mesenchymal cell is observed and this has implications on osteoblasts for expressing receptor activator of nuclear factor-$_K$B ligand (RANKL) that affects osteoclast differentiation. In vitro studies show that TNF-α increases mesenchymal cell proliferation and bone mineralization [18], [19].

The limitation of this study includes blood serum TNF-α level measurement that involves many other factors that may influence the measurement results. Further studies need to be done to assess TNF-α expression in wound or bone defect areas using more specific examinations such as immunochemical examinations or PCR. Histological analysis and analysis on other cytokines need to be studied to be able to explain the role of collagen membrane in wound healing and bone regeneration.

5. Conclusions

Based on the explanation on the study findings, the use of collagen membrane can reduce the level of TNF-α in blood serum. This reduction of the pro-inflammatory cytokine indicates that collagen membrane has a potential to accelerate wound healing.

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