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The influence of collagen membranes and tetracycline on the PDGF-BB expression and osteoblast amount in bone defects healing: Experimental study in mice

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Abstract : Introduction: Collagen membranes have been used in Guided Tissue Regeneration and Guided Bone Regeneration methods. Collagen membranes immersed in a tetracycline solution before the application is able to slow down the degradation of the membrane. Tetracycline is an antibiotic that has widely used in periodontal treatment, with an antibacterial effect that inhibits an MMP matrix which giving therapeutic advantages in the regenerative procedures. The objective of the study was to evaluate the effect of collagen and tetracycline membranes on the tissue regeneration by analyzing the PDGF-BB expression and osteoblast cells in the bone healing process. **Methods:** This study was an experimental study conducted on 48 male Wistar rats, divided into four treatment groups. The P1 group was the group with collagen membrane added with tetracycline; the P2 group was with collagen membrane collagen without tetracycline; the P3 was without any collagen membrane or tetracycline; the P4 group was without membrane but with tetracycline. Animal testing was conducted to see the effect of collagen and tetracycline membranes on wound healing by measuring the PDGF-BB expression, and the number of osteoblasts. Bone defects were made in the mandibular bones of mice, the PDGF-BB expression was analyzed by immunohistochemistry, and the histologic number of osteoblasts was analyzed with HE histological preparations. The examination or measurement of these parameters was performed on the third, seventh, fourteenth, and twenty-first days. All data were collected and analyzed statistically by using the ANOVA and t-test. **Results:** From the statistical analysis were obtained that the PDGF-BB expression among the treatment groups was $p = 0.0003$; the number of osteoblasts $p = 0.001$. The results of the correlation analysis of the PDGF-BB expression with osteoblast number was $p = 0.001$; $r = 0.64$. **Conclusion:** Collagen membrane with tetracycline was able to increase the PDGF-BB expression and the number of osteoblasts in the wound healing process.

Keyword : Collagen membrane, PDGF-BB expression, wound healing, osteoblast.

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Introduction

The primary goal of periodontal therapy is the regeneration of the dental support structures and stopping the disease progression.[1] In the guided tissue regeneration and guided bone regeneration methods, the barrier membrane was used to prevent unwanted cells around the defects to stimulate periodontal regeneration.[1,2] The resorbable membrane should facilitate the attachment, proliferation, and migration of cells on the membrane surface and cover the defects from any oral bacteria.[3] The connective tissue cells attachment to the inner surface of the membrane stimulates periodontal regeneration and attachment, helping to stabilize blood clots and membrane integration into the tissues.[3,4]

Collagen is selected as one of the materials manufacturing the resorbable membranes.[4] Collagen also has biological activities that play an important role in the formation of coagulum, chemotaxis, and activating the neutrophils and fibroblast cells of periodontal ligaments and gums, with low immunogenicity properties.[4,5] One of the disadvantages of collagen is a rapid degradation within days to weeks, duration of membrane barrier function is important for a successful treatment.[5,6] The integrity loss of the collagen membrane structure due to the rapid biodegradation process by macrophages and polymorphonuclear leukocytes (PMN) through enzymatic activity becomes the major problem in resorbable collagen membrane type.[6,7] The effectiveness of the resorbable membrane showed some limitations in tissue regeneration, and further clinical research was needed to assess its role in bone regeneration. The resorbable membrane has the potential to support bone formation only with enough time to function as a barrier over a certain period.[7,8]

Many ways are done to improve the stability of the collagen membrane material to make a longer degradation time, thus having sufficient time to function as a barrier. The degradation of collagen membranes caused by collagenase activity produced by matrix metalloproteinases(MMPs).[8,9] One effort to improve membrane stability is by adding tetracycline antibiotics as the anti-collagenase to inhibit the formation of MMPs, and to reduce the effect of bacteria that may arise during surgical procedures.[9] Tetracycline is a broad-spectrum antibiotic that able to inhibits all gram-negative or positive bacteria.[10] The effect of tetracycline is very useful in the early phase of the wound healing and may also improve the treatment outcomes.[11] The study conducted by Moses et al. stated that the collagen membrane immersed in the tetracycline liquid before implantation was able to significantly slowing down the degradation of the collagen membrane.[11]

Platelet-derived growth factor (PDGF) plays an important role in all phases of wound healing. Various cells secrete PDGF, namely fibroblasts, endothelial cells, smooth muscle cells, platelets, and inflammatory cells.[12] PDGF is one of the growth factors that play a role in regulating cell division and growth. PDGF affects various types of cells that play a role in wound healing, mitogenic stimulation, fibroblasts and smooth muscle cells chemotaxis, neutrophil and macrophages chemotaxis, and stimulating the production of other important growth factors in wound healing.[12,13] PDGF able to stimulate various matrix molecules such as fibronectin, collagen, proteoglycans, and hyaluronic acid. PDGF also plays an important role in the advanced stages of wound healing, stimulates collagen matrix in-vitro, stimulates fibroblasts to produce or secrete collagenases, and also plays a role in the remodeling phase of wound healing.[12,14]The objective of the study was to analyse the effect of collagen and tetracycline membranes on PDGF-BB expression and the amount of the osteoblast cells in the bone healing process.

Materials and Methods

The study was conducted in the Animal Hospital of Institut Pertanian Bogor. The protocol and treatment of animals have been approved by the animal care and use committee in the Faculty of Veterinary Institut Pertanian Bogor (IPB). This study was an in-vivo study towards male Wistar rats, aged 2-3 months, with the weight of 250-300 grams. A total of 48 rats were divided into 4 groups: Group P1 is consisted of collagen membranes + tetracycline, group P2 is consisted of collagen membranes without tetracycline, group P3 is consisted without membrane and tetracycline, group P4 is consisted of tetracycline without membrane.. In the P1 group, the collagen membrane was immersed in a solution of 50 mg/mL tetracycline before covered the bone defects of the rat jaw. Each group was consisted of 12 mice and analysedfour times, at the third, seventh, fourteenth, and twenty-first days (3 rats in each analyse period).

Collagen Membrane Preparation.

Collagen was extracted from the collagen of white snapper scales using 0.5 M of acetic acid.[15]The wet collagen was freeze-dried to obtain a dry collagen. Collagen mixed with chitosan and made in the form of membrane sheet with the membrane thickness of 0.6 mm.

Surgical Procedure.

As much as 48 Wistar rats were obtained from local vendor farms in the city of Bogor.Anesthetic action was performed by intraperitoneal injection using 75 mg/kg BW of Ketamil® and 10 mg/kg BW of Xylazil®. The incision was done extra orally with no. 15 knife in the buccal region followed by blunt dissection until reached the mandibular angle. Establishment of defected mandibular bone in the 2 mm diameter mandibular angulus region was done using low-speed the 2 mm diameter bur followed by 0.9% NaCl irrigation. The placement of the collagen membrane covered the bone defect. Closure of the incision wounds was done with the suture using 5.0 nylon yarn. Each group was performed necropsies towards 3 rats each time, on the third, seventh, fourteenth, and twenty-first days, with exsanguination under the influence of anesthesia.

Immunohistochemistry of PDGF-BB

Deparaffinization of warmed tissues sections in 3 changes of slide bite for 3 minutes each, hydrate slides in s graded series of alcohol (100%, 95%, and 70%). As much as 4 drops of peroxidase (endogenous peroxidase blocker) were applied, then incubated for 5 minutes. The slide was heated with the microwave for 3 minutes, then chilled for 20 minutes. Afterwards, the slides were dried and applied as much as 4 drops of Biocare® Background Sniper (protein blocker) then settled for 15 minutes at the room temperature. The protein blocker then drained and applied as much as 4 drops of the appropriate primary antibody and settled overnight. Afterwards, the slide washed for 2 times with the PBS washes buffer for 2 minutes each. The Trekkie® Universal Link was applied for as much as 4 drops after, and incubated for 20 minutes at the room temperature, then washed for 2 times with the PBS washes buffer for 2 minutes each. The TrekAvidin®-HRP was applied for as much as 4 drops after, and incubated for 10 minutes at the room temperature, then washed for 2 times with the PBS washes buffer for 2 minutes each. The Betazoid® DAB Chromogen solution was applied for as much as 4 drops after, and settled for 10 minutes at the room temperature, then washed with deionized water, and added as much as 4 drops of CAT® Hematoxylin and settled for 10 minutes. Washed in tap water afterwards and rinsed in deionized water, then dried with 100% of alcohol that changed 3 times and cleaned with 3 times changed xylene.

Histological and Immunohistochemical Assessment

The assessment of PDGF-BB expression in histologic immunohistochemistry preparations was performed by observing the number of brown cells indicated a PDGF-BB growth factor. The calculation of PDGF-BB expression was performed with five microscope field view under 40X magnification around the bone defects. Histologic examination was performed with four microscope field view under 40X magnification along the bone defects to analyse the number of osteoblast cells.

Statistical Analysis.

Presented in the average value ± SD in each group. The difference was statistically analysed using one-way analysis of variance (ANOVA) followed by the t-test. The correlation between PDGF-BB with osteoblasts was tested by the Pearson correlation, with the significant difference determined if $p<0.05$.

Results

Table 1 showed that there was a difference of PDGF-BB expression amongst all four treatment groups with p=0.0004 ($p<0,05$). The PDGF expression was generally the same, and culminated at the 7^{th} day 7, and tend to decrease over the healing time. The highest value was seen in the collagen membrane + tetracycline group (P1) at the 7^{th} day, and the lowest value was seen in the group with no collagen membrane and tetracycline (P3) at the 3^{rd} day.

The next stage was the difference test of the average value of PDGF-BB based on treatment healing time factors (**Table 2**). Based on the t-test results showed that there was a significant difference of PDGF-BB expression between the group with collagen membrane + tetracycline (P1) with the group without membrane and tetracycline (P3) (p>0,05). This difference suggested that the application of collagen membrane affected the PDGF-BB expression. On the seventh day, the PDGF-BB expression in the group with collagen membrane and tetracycline was higher than the group with collagen membrane without tetracycline, although statistically insignificant (p=0.12), but tend to show higher PDGF-BB expression.

Table 1. Difference of PDGF-BB expression among all treatment groups

PDGF-BB expression	Treatment				P-Value
	P1 (n=12)	P2 (n=12)	P3 (n=12)	P4 (n=12)	
3rd day Mean (SD)	31 (2.0)	32 (3.61)	23 (2.52)	29 (4.73)	0.0004
7th day Mean (SD)	43 (4.73)	38 (1.0)	29 (2.52)	33 (4.16)	
14th day Mean (SD)	32 (5.51)	34 (7.55)	29 (1.15)	27 (4.73)	
21st day Mean (SD)	33 (2.08)	32 (5.69)	29 (1.53)	26 (5.57)	

Notes: Countable f value = 4.05
P1 = Collagen membrane (+) tetracycline (+)
P2 = Collagen membrane (+)tetracycline (-)
P3 = Collagen membrane(–) tetracycline (-)
P4 = Collagen membrane (-) tetracycline (+)

Table 2. PDGF-BB difference test results based on treatment and healing time factors

Time (day)	P1 - P2	P1 – P3	P1 – P4	P2 – P3	P2 – P4	P3 – P4
3rd day	0.69	0.01*	0.60	0.02*	0.48	0.09
7th day	0.12	0.01*	0.04*	0.005*	0.97	0.30
14th day	0.68	0.51	0.29	0.34	0.22	0.39
21st day	0.65	0.03*	0.09	0.42	0.28	0.46

***Significant p-value**

Table 3. Difference of osteoblasts amount among all treatment groups

Osteoblasts	Treatment				P-Value
	P1 (n=12)	P2 (n=12)	P3 (n=12)	P4 (n=12)	
3rd day Mean (SD)	24.3 (3.51)	21.3 (1.53)	16.3 (1.53)	18.3 (1.53)	0.001
7th day Mean (SD)	41.7 (7.51)	32.3 (3.51)	26.7 (1.53)	27.7 (1.53)	
14th day Mean (SD)	32 (2.00)	30 (2.65)	21 (1.00)	24.3 (1.53)	
21st day Mean (SD)	25 (1.73)	28 (3.00)	18 (2.00)	19 (2.65)	

Notes: Countable f value = 16.17

Table 3 showed the difference of the amount of osteoblasts amongst all treatment groups with p=0.001 (p<0.05). The highest amount of osteoblasts was seen in the collagen membrane + tetracycline group (P1) at the seventh day, and over the healing time, the amount of osteoblasts were decreased in all treatment groups (**Figure 1**).

The next stage was performed the post-hoc analysis and a difference test of theaverage amount of osteoblasts based on treatment and healing time factors (**Table 4**). Based on the t-test results showed that there was a significant difference of the osteoblast amount between the collagen membrane + tetracycline group (P1) with the group without collagen membrane and tetracycline (P3) (p<0,05). Significant differences were also seen between the P1 and P4 and the P2 and P3.

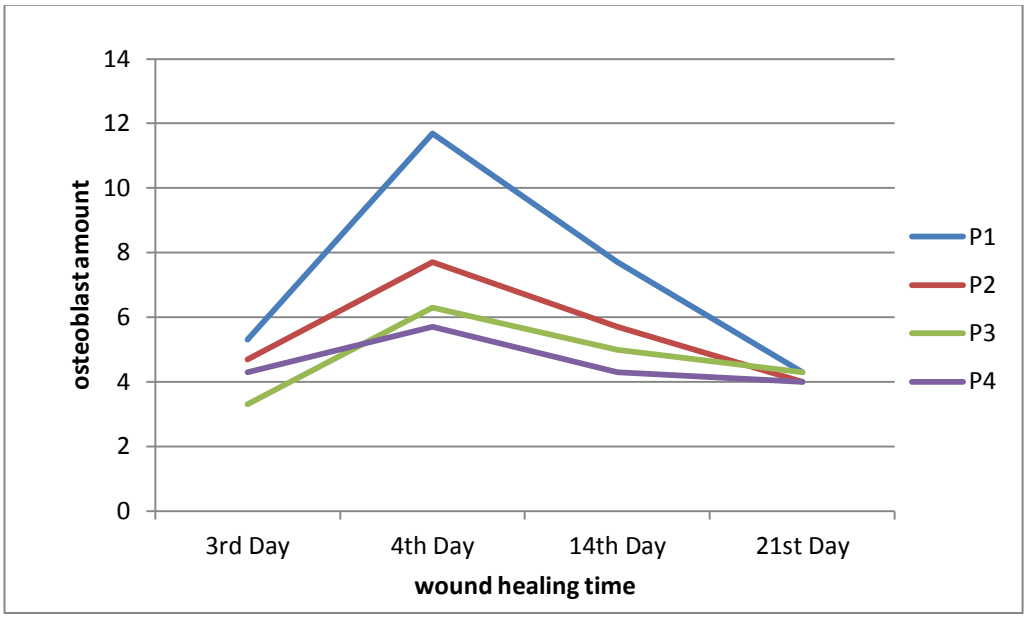

Figure 1.The number of osteoblasts in all treatment groups Based on wound healing time

Table 4. Difference of osteoblasts amount test results based on treatment and healing time factors

Time (day)	P1 - P2	P1 – P3	P1 – P4	P2 – P3	P2 – P4	P3 – P4
3rd day	0.25	0.02*	0.05*	0.01*	0.07	0.18
7th day	0.12	0.02*	0.03	0.06	0.10	0.46
14th day	0.35	0.001*	0.006*	0.005*	0.03*	0.03*
21st day	0.20	0.01*	0.03*	0.008*	0.017*	0.62

***Significant p-value**

Table 5. Correlation between PDGF-BB with Osteoblasts

Correlation	r	r^2	p-value	Relationship level according to Guilford Criteria
PDGF-BB with osteoblasts	0.68	0.462	0.001	Strong

Notes: r = correlation
r^2 = coefficient of determination

The correlation of PDGF-BB expression with osteoblasts can be seen in **Table 5**. Based on the Pearson correlation test showed that there was a significant correlation between PDGF-BB and osteoblasts amount with p=0,001. Regression analysis result showed that the correlation of PDGF-BB correlation with osteoblast was 46.2% (r=0.68).

Discussion

This study showed a significant differencein the PDGF-BB expression amongst all four treatment groups. The use of collagen membranes was able to affect the PDGF-BB expression in the wound healing process. Collagen membranes applied to defected bone and incision wounds may help to accelerate the healing

process by the presence of the growth factor parameters, especially the PDGF-BB expression in the area of defected bone and incision.

The increasing PDGF-BB expression in the group with applied with collagen membranes was occurred due to the collagen effect towards the platelet aggregation and its ability to bind fibronectin. Collagen also has the ability to facilitates the fibroblasts and osteoblasts chemotaxis and also the attachment, proliferation, and differentiation of various cells that contribute to the wound healing process. The exact mechanism of collagen interaction has not fully known, but the exact data indicated that the interactions of collagen and platelets are the initial stage of the healing process.[16,17] The hemostasis ability of collagen was indicated by the fact that the time of exsanguination will prolongedin cases with abnormal collagen.

PDGF has been reported to play an important role in regulating the proliferation and migration of osteoblast and fibroblast cells. The results showed that the PDGF-BB expression was positively correlated with the amount of osteoblasts. The higher the PDGF-BB expression, the higher amount of osteoblasts found. This result was consistent with the research conducted by Nisteret al. which discovered that PDGF-BB was able to stimulate the proliferation and differentiation of fibroblast and osteoblast cells.[18]

In periodontal therapy, various growth factors have been studied and observed their role in the regeneration of periodontal tissue. One of the significant growth factors based on its role in the regeneration of periodontal tissue is PDGF-BB. In-vivo studies have demonstrated that a direct PDGF-BB application towards the defected bone area enhanced the granulation tissue formation. Subsequent studies showed the increase of the wound healing after a local PDGF-BB application in the mouse skin incision wounds, the rabbit ears excision wounds, and periodontal lesions in dogs and monkeys. The PDGF-BB application also increased the wound strength from 150% to 170%, accelerated the healing time, and also improved the reepithelization and neovascularization.[14,19]

Various studies have shown that the addition of PDGF-BB was significantly increased the proliferation and differentiation of periodontal tissue cells.[94] A study conducted by Denison et al. showed that the addition of PDGF-BB hadstimulatedmitogenic and chemotaxis of the fibroblasts in the periodontal ligament and gingival.[20] The research of Oates et al. towards the periodontal ligaments cell culture resulted in the cell division stimulation after the addition of PDGF-BB.[21] Another study conducted by BartoldandReben using an in-vitro wound model suggested that the addition of PDGF-BB may increase the wound filling.[22] These studies showed that PDGF-BB was a regulator that plays an important role in the response of periodontal wound healing.

The results of the statistical test on the comparison of osteoblasts amount showed a significant difference compared with the group without collagen membrane and tetracycline (P3), or with the group without collagen membrane but applied with tetracycline (P4). This result showed the role of the collagen membrane on increasing the amount of osteoblasts was stronger than the tetracycline.

A membrane barrier ideally has the ability to facilitates the cell attachment and induces the migration of progenitor cells. The important thing for the success of a membrane barrier is the initial cell attachment towards the membrane which will go through the cellular replication stage afterwards. This attachment process consists of the adsorption of glycoproteins on the membrane surfaces, cell contacts, attachments, and spreading. The membranes used should not have any damaging effect on the cell and have the capacity to promotes the cell growth and proliferation for such processes to take place.[23] Collagen consists of basic molecules such as lysine and arginine, and the site of specific cell attachment, which is arginine-glycine-aspartate (RGD). RGD actively stimulates cellular attachment by binding to the integrin receptors,andthis bond plays an important role in cell growth and differentiation, and also the overall regulation of the cell function.[25]

The results showed that the collagen membrane was able to increase the number of osteoblast cells. These results were consistent with previous research conducted by Wang et al., S Tangakumara et al., and Adrian Kasaj et al. The research conducted by Wang et al. was an in-vitro study stated that the collagen membrane was able to facilitate the cell attachment and the formation of osteoblast cell layers that will ultimately improve the bone regeneration.[24] A study conducted by Tangakumaraet al. showed that the initial adhesion attachment of osteoblasts occurred after 24 hours. This interval time showed the attachment of the

mesenchymal cells towards the higher membrane. The osteoblast attachment on the membrane barrier was significantly higher in the collagen membrane made from the type I collagen.[25]

A study conducted by Adrian Kasaj et al. suggested that the collagen membrane increased the proliferation of the gingival and periodontal ligaments fibroblast cells compared to the synthesis membrane (PTFE membrane).[26] Membrane matrices composed by collagen and chondroitin glycosaminoglycans promoted the cell proliferation and extracellular macromolecular accumulation. It was also stated that non-resorbable properties of the PTFE membranes inhibited the synthesis of the gingival fibroblasts DNA and caused a decrease in collagen and glycosaminoglycans synthesis which were a major component of the extracellular matrix.[26]

Tetracycline is a broad-spectrum antibiotic that affects the bone metabolism. The administration of tetracycline in monkeys during the 17-days period have increased the osteoblasts activity and osteoid formation in alveolar bone. Based on the animal studies, tetracyclines also affects the bone deficiency disease. Tetracycline may inhibit collagenase or collagen decomposition, and therefore, affects the collagen metabolism, the formation of collagen connective tissue on the apical part of the periodontal pocket after the patient received a subgingival of doxycycline antibiotics. This condition occurred due to a stable concentration of collagen matrices that affected the proliferation and differentiation of osteoblasts.[27]

The Increasing of the osteoblast proliferation and differentiation after tetracycline administration havebeen observed in diabetic rats, with the results of the new bone formation and improvement in osteopenia. Tetracycline has also been reported of improving the osteoblast structure. Research towards diabetic rats resulted in the increase of the cytoplasm organelles required for protein synthesis (Golgi-RER system) and active transport (mitochondria). Other studies have reported that in the tetracycline concentrations of 60-80 mg/ml may inhibit the osteoblast proliferation, possibly along with a mitochondrial damage. Administration of tetracycline at low doses (less than 50 mg/ml) may induce osteogenesis, but high concentrations (more than 50 mg/ml) may inhibit the osteogenesis processes.[28]

Conclusion

Collagen membranes and tetracycline affected the wound healing process by increasing the PDGF-BB expression and osteoblast amount. Further research needed with longer research time to observe the role in the wound healing process by evaluating the formation of soft tissue and bone.

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