

Antiinflammatory Effects of Tetracycline - loaded Biodegradable Membranes in Experimental Periodontitis of Beagle Dogs

Hyung - Sik Jun¹ · Yang - Jo Seol¹ · Yoon - Jeong Park² · Yong - Moo Lee¹ · Young Ku¹

In - Cheul Rhyu¹ · Seung - Jin Lee² · Soo - Boo Han¹ · Sang - Mook Choi¹
Soo - Kyoung Kwon³ and Chong - Pyoung Chung¹

¹Dept. of Periodontology, College of Dentistry, Seoul National University,

I. Introduction

The ultimate objective of periodontal therapy is the regeneration of the periodontium destroyed by inflammatory periodontal disease. This means the formation of new connective tissue attachment with connective tissue fibers inserting into diseased root surface with formation of new cementum and bone regeneration. Guided tissue regeneration(GTR) provides environment for regeneration of cementum, periodontal ligament, and bone by placing a barrier membrane between the gingiva and the diseased root surface¹⁻³). The purpose of the barrier membrane is not only to prevent proliferation of gingival epithelium and connective tissue into the dentogingival wound space, but also to divert mechanical stress from the coagulum - tooth interface, and thus allow undisturbed organization of the blood clot and promote early attachment of connective tissue elements to the root surface⁴).

Post - operative contamination of the membrane or infection of the surgical site is a problem often encountered⁵) using barrier membrane for GTR therapy. This is especially true when the membrane is exposed⁶). Clinically, many techniques have been tried to prevent the exposure of the membrane. But the exposure of the membrane due to the morphology of the tooth itself⁷), incomplete coverage of the membrane by the gingival flap⁸), dehiscence of the gingival flap⁹), or gingival recession¹⁰) is a common complication resulting from guided tissue regeneration therapy. This increases incidence of infection of the newly formed tissue beneath the exposed membrane when it is exposed¹¹). Microorganisms can adhere to and colonize exposed membranes leading to the development of a nidus of infection. Microbial adherence has been associated with infection and subsequent rejection of many biomaterials¹²). Increased bacterial contamination of the site where a membrane was

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used could be seen⁶). Some have reported that e - PTFE membranes show higher incidence of bacterial infection^{4,13 - 15}). Infections of the membranes affect clinical outcomes. Many studies have shown that contamination of the membranes and the degree of infection are related to decreased clinical attachment gain^{10,16 - 18}). It has been reported that clinical healing response is related to whether the membrane is infected or not¹⁹). This explains the importance of infection control as a part of regenerative attempt²⁰).

In many researches, systemic antibiotics has been administrated to prevent post-operative infection after guided tissue regeneration therapy^{21 - 26}). Tetracycline is effective on a wide range of periodontal pathogens^{27 - 32}), and inhibits connective tissue destruction by inhibiting neutrophil collagenases³³). Due to these effects, tetracycline has been used systemically or locally for treatment of periodontal diseases³⁴). Tetracycline binds to the root surface and is slowly released from the root surface³⁵). Reattachment or regeneration of the periodontal tissues may also be enhanced by promotion of fibroblast attachment, conditioning of root surfaces, and inhibition of collagenase activity^{36 - 38}). Also tetracycline can increase attachment³⁹), inhibit bone resorption in vitro^{37,40}), and increase collagen formation of osteoblasts⁴¹). Pretreatment of dentin with tetracycline increased migration of periodontal ligament cells. Also fibronectin adhered better to the root surface when the root was treated with tetracycline⁴²).

In this study, membrane was formed by polyglycolide mesh coated on 10% tetracy-

cline(TC) containing polylactic acid. The purpose of this study is to evaluate anti-inflammatory and antimicrobial effects of Tc-loaded biodegradable membrane in experimentally induced periodontitis in beagle dogs and to investigate release characteristics of the Tc-loaded membrane.

II. Materials and Methods

1. Formation of the Tetracycline-loaded Membrane

The membrane used in this experiment was formed as below⁴³). PGA meshes were knitted with a tube knitter(Koike Ltd., Nara, Japan) with PGA(polyglycolic acid, viscosity 18,000 poise, Samyang Co., Seoul, Korea). Initial strength of the mesh was 5.2g/d. The mesh was spread out, fixed and covered with polylactic acid(MW 300,000, Purac Biochem BV, Gorinchem, Holland). This was dried for 24 hours at room temperature and the was evaporated. This was vacuum dried for 24 hours and the remaining solvent was removed. To produce a membrane containing tetracycline, first, polylactic acid was dissolved in methylene chloride and ethylacetate was added. Tetracycline(Sigma Chemical Co., St Louis, MO, USA) was mixed with dissolved polylactic acid at 10% weight ratio. PLA solutions were cast on PGA meshes by using a doctoring blade and solvents were evaporated in air for 24 hours and further dried under vacuum for 24 hours to remove residual solvents. Membranes used in the experiment was sterilized with EO gas one day before the experiment.

2. Experimental Animals

Healthy beagle dogs weighing approximately 15 Kg were used regardless of sex. Of the six, five beagle dogs were used to evaluate the efficacy of the membrane containing tetracycline on guided tissue regeneration. Three dogs were assigned to the test group which was implanted with tetracycline - loaded membrane (Group I). Two dogs were assigned to the membrane without tetracycline (Group II) and no membrane used (Group III) contralaterally. To prevent the influence of tetracycline, tetracycline - loaded membranes were used in the test group only. One remaining dog was used to evaluate the concentration of tetracycline released into the gingival sulcus.

3. Defect Formation

Bony defects were formed 2 - 3 months before membrane placement. General anesthesia was induced in experimental animals by infiltration of equal parts of 2% Xylazine hydrochloride (Rumpun, Bayer Korea, Korea) and ketamine hydrochloride (Ketalar, Yuhan, Korea). Local anesthesia was obtained by infiltration of 2% lidocaine containing 1:100,000 epinephrine for bleeding control. Upper canine and 2nd premolar, or lower 3rd and 4th premolars were used in the experiment. Full thickness flap was raised after sulcular incision was extended one tooth mesial and distal to the test tooth. Vertical incision was added when necessary. Alveolar bone defects were formed with low speed carbide round bur

and chisel after removal of all granulation tissues and calculus. Class II furcation defects were formed in premolars according to following dimensions; 5mm vertically from CEJ, mesiodistally to the line angle, buccolingually 2 - 3mm deep. In canines, dehiscence defect measuring 4mm wide and 5mm deep from neighboring alveolar crest was formed. To prevent natural healing and to induce chronic inflammation, alveolar bone defect was filled with silicone rubber impression material (EXAMIX™, GC America Inc., Japan) and sutured with black silk. Penicillin G procaine (Pfizer Co., New York, U. S. A.) was injected for 5 days intramuscularly and soft fluid diet was given for 2 weeks. Stitch out was done 2 weeks after operation.

4. Surgical Procedure

2 - 3 months after defect formation, general anesthesia was induced according to the same procedure as described previously. Full thickness flap was raised including one tooth mesial and distal to the test area with sulcular incision. Scaling and root planing was done after removal of rubber impression materials. To prevent cross-over effect, only tetracycline - loaded membranes were used in the test group. The membrane was trimmed to cover the defect 2 - 3mm over the bone. The membrane was sling sutured on the tooth and flap was repositioned. Complete coverage of the membrane was attempted. Penicillin G procaine (Pfizer Co., New York, USA) was injected intramuscularly for 7 days and soft fluid diet was given for 2 weeks. Oral

hygiene was maintained with 0.1% chlorhexidine three times a week from post - op 2 weeks. Stitch out was done post - op 2 weeks.

In two weeks postsurgery, the dogs were sacrificed. Maxilla and mandibles were block - resected and fixed in 10% formalin for histologic preparation and analysis. Undecalcified specimens were prepared according to Donath and Breuner ' s method⁴⁴).

5. Measurements of Clinical Parameters and Microbiological Assay

Following clinical parameters were measured directly before surgery, and 1, 2, 4 weeks post - operatively. GI(Gingival Index, Loe & Silness 1963)⁴⁵), PI(Plaque Index, Silness & Loe 1964)⁴⁶) of the test site were measured. Periopaper strip (Proflow Inc., NY, USA) was inserted in the gingival sulcus for 30 seconds to sample gingival crevicular fluid and GCF was measured with Periotron 8000 (Proflow Inc., NY, USA).

Microbiological assay was done after measurement of clinical parameters. To sample subgingival plaque three paper points(DiaDent Group International, Chongju, Korea) were inserted in the pocket for 30 seconds and these were placed in Moller ' s VMGA III and immediately moved to the anaerobic chamber(Forma, OH, USA), and mixed with a vortex mixer(Vortex mixer) for 30 seconds. Serial dilution of the VMGA III solution containing bacteria was done to 1/10, 1/100, 1/10000 of the original solution. 100 μ l of this solu -

tion was spreaded on selective agar plates. Aerobic bacteria was plated on 5% sheep blood agar plate and cultured in a 10% CO₂ chamber at 37 (Vision, Korea), and anaerobic bacteria was plated on Tryptic soy agar plate containing 5% sheep blood, vitamin K, and hemin and cultured in an anaerobic chamber containing H₂, 10% CO₂, 80% N₂ at 37 . After 7 days, the total number of CFU of aerobic bacteria and anaerobic bacteria was calculated.

6. Bioassay of Released Tetracycline

Tetracycline - loaded membranes were placed on left and right upper canines and 2nd premolars, and on lower 2nd and 3rd premolars according to the above procedure. Method of Bennet et al^{47 - 49}) was modified to investigate the concentration of tetracycline in the tissues. At post - operatively 1, 3, 5, 7, 14 days, Periopaper strip (Proflow Inc., NY, USA) was placed between the gingiva and the membrane for 30 seconds to detect tetracycline in tissue fluid. These periopapers were placed on a B. cereus cultured nutrient agar plate and cultured for 36 - 48 hours at 37 . After culture, inhibitory zones were measured with a caliper placed perpendicular to the periopaper and measured to the nearest mm. Standard curve was calculated by serial dilution of tetracycline. The concentrations of the tetracycline released in the gingival sulcus were measured indirectly with standard curve.

7. Statistical Analysis

Table 1. Comparison of GI of the three groups (mean \pm SE)

	Baseline	1 week	2 weeks	4 weeks
Group I	1.58 \pm 0.15	1.83 \pm 0.13	1.08 \pm 0.19*	1.17 \pm 0.21*
Group II	1.25 \pm 0.25	1.75 \pm 0.48	1.50 \pm 0.50	1.50 \pm 0.25
Group III	1.75 \pm 0.48	1.50 \pm 0.29	1.75 \pm 0.25	1.50 \pm 0.25

* : The mean difference is significant at the 0.05 level from baseline.

Group I : tetracycline - loaded membrane group

Group II : tetracycline - unloaded membrane group

Group III : without membrane group

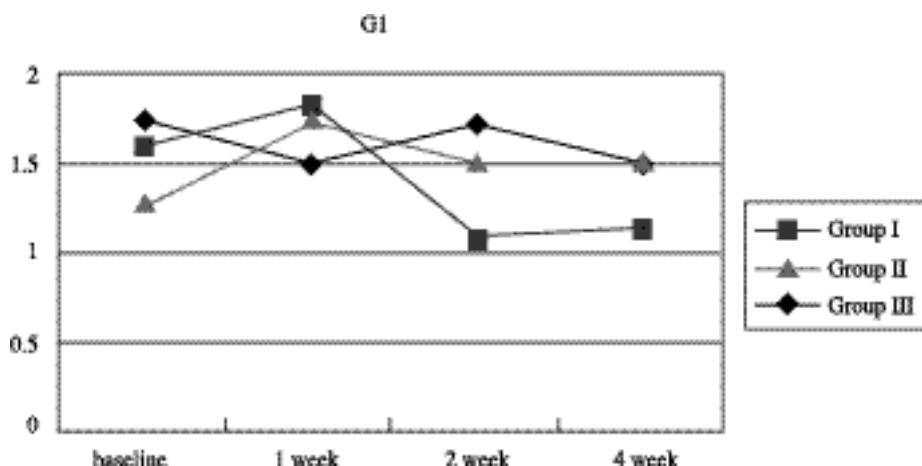


Figure 1. Comparison of GI of the three groups. GI of the Group I at post - op 2 and 4 weeks showed significant decrease from baseline. GI of the Group II and the Group III showed slight

Table 2. Comparison of PI of the three groups(mean \pm SE)

	Baseline	1 week	2 weeks	4 weeks
Group I	2.00 \pm 0.15	1.58 \pm 0.13*	1.50 \pm 0.19*	1.42 \pm 0.21*
Group II	1.50 \pm 0.50	2.00 \pm 0.00	1.75 \pm 0.48	1.75 \pm 0.48
Group III	2.00 \pm 0.41	2.00 \pm 0.00	1.50 \pm 0.29*	1.25 \pm 0.25*

* : The mean difference is significant at the 0.05 level from baseline.

Sample size was calculated on a tooth basis. Homogeneity test of standard deviation was done on each group at each period to assess whether application of parametric analysis is appropriate. Repeated measures ANOVA method was used to analyze the effect of treatment, and to compare the

interactions of treatment method and time. For posthoc multivariate comparison, Dunnett test was done. 0.05 was taken for error. SPSS software version 7.0 (SPSS Inc., Chicago, IL. USA) was used for statistical analysis.

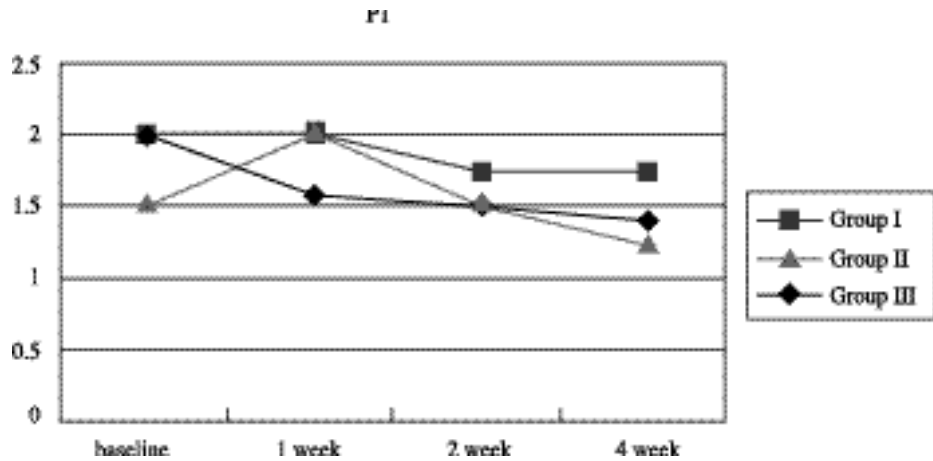


Figure 2. Comparison of PI of the three groups. Plaque index of the Group I and the Group III decreased. In the Group II, Plaque index increased.

Table 3. Comparison of GCF volume of the three groups(mean \pm SE)

	Baseline	1 week	2 weeks	4 weeks
Group I	80.83 \pm 6.60	104.83 \pm 5.85	93.42 \pm 7.05	86.25 \pm 7.30
Group II	74.00 \pm 14.39	99.50 \pm 25.47	82.25 \pm 5.81	76.00 \pm 8.42
Group III	73.25 \pm 26.16	119.25 \pm 15.43	80.50 \pm 27.73	67.00 \pm 12.25

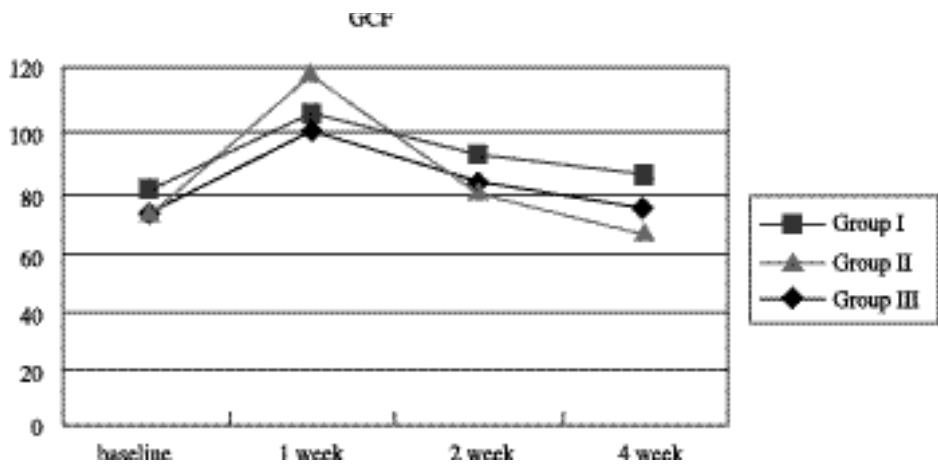


Figure 3. Comparison of GCF volume of the three groups. Post - op 1 week values in all three groups are significantly increased from baseline. Thereafter GCF volumes decreased with time to a level similar to baseline measurements at week 4.

III. Results

1. Baseline examinations

Difference in dependent variables(GI, PI,

Table 4. Total CFU of anaerobic bacteria(mean ±SD)

	Baseline	1 week	2 weeks	4 weeks
Group I	6.31 ± 0.21	4.44 ± 0.24 ⁺ *	5.41 ± 0.27 [*]	5.31 ± 0.38 [*]
Group II	6.29 ± 0.23	6.21 ± 0.38	6.04 ± 0.43 [§]	5.80 ± 0.44 [§]
Group III	6.30 ± 0.31	5.52 ± 0.45	5.32 ± 0.37 [*]	5.02 ± 0.44 [*]

CFU of anaerobic bacteria was expressed as log₁₀ of the value.

*: The mean difference was significant at the 0.001 level from baseline.

+: The mean difference was significant at the 0.001 level between Group I and group II, III.

§: The mean difference was significant at the 0.001 level between Group I, III and group II.

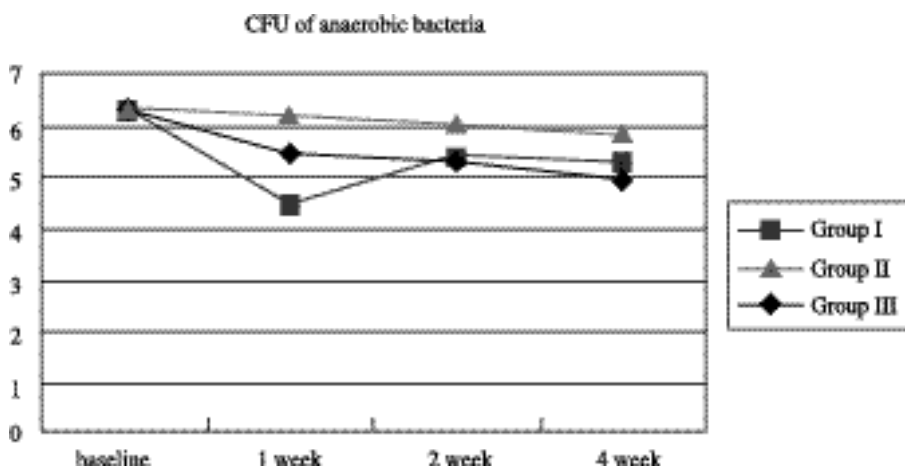


Figure 4. Total CFU of anaerobic bacteria. CFU of bacteria was expressed as log₁₀ of the value. Total CFU of the Group I was less than that of the Group II throughout the study period.

Table 5. Total CFU of aerobic bacteria(mean ± SD)

	Baseline	1 week	2 weeks	4 weeks
Group I	5.31 ± 0.27	4.41 ± 0.20 ⁺ *	5.20 ± 0.31	5.26 ± 0.37
Group II	5.37 ± 0.16	5.51 ± 0.21	5.40 ± 0.19	5.32 ± 0.26
Group III	5.39 ± 0.25	5.35 ± 0.22	5.25 ± 0.24	5.43 ± 0.31

CFU of anaerobic bacteria was expressed as log₁₀ of the value.

*: The mean difference was significant at the 0.001 level from baseline.

+: The mean difference was significant at the 0.001 level between Group I and group II, III.

GCF, total colony forming units of anaerobic bacteria, total colony forming units of aerobic bacteria) were not statistically significant at baseline among groups.

2. Clinical measurements

GI in the membrane group(Group I, II) showed a slight tendency to increase at 1 week after surgery. In the Group I, this decreased significantly 2 weeks after surgery ($p < 0.05$) and this was maintained

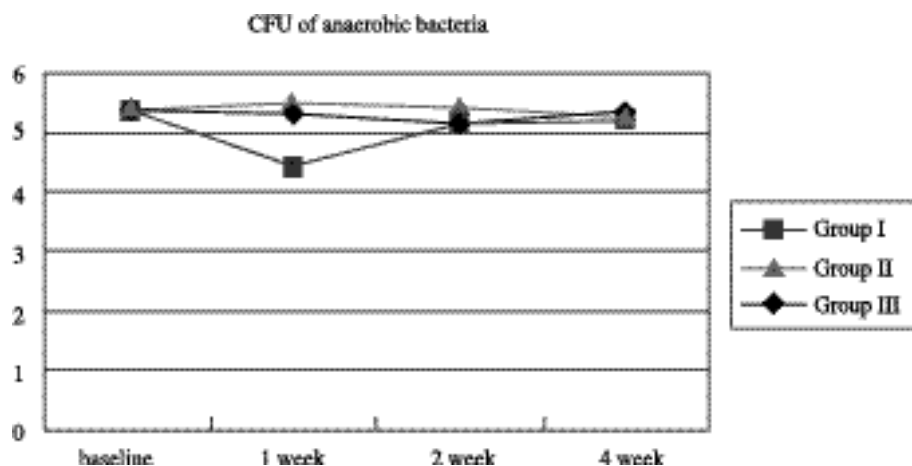


Figure 5. Total CFU of aerobic bacteria. CFU of bacteria was expressed as log₁₀ of the value.

Table 6. Tetracycline release kinetics

days	1 day	3 days	5 days	7 days	14 days
concentration (µg/Ml)	52.50 ± 14.40	35.00 ± 13.42	30.83 ± 14.63	29.17 ± 13.20	—

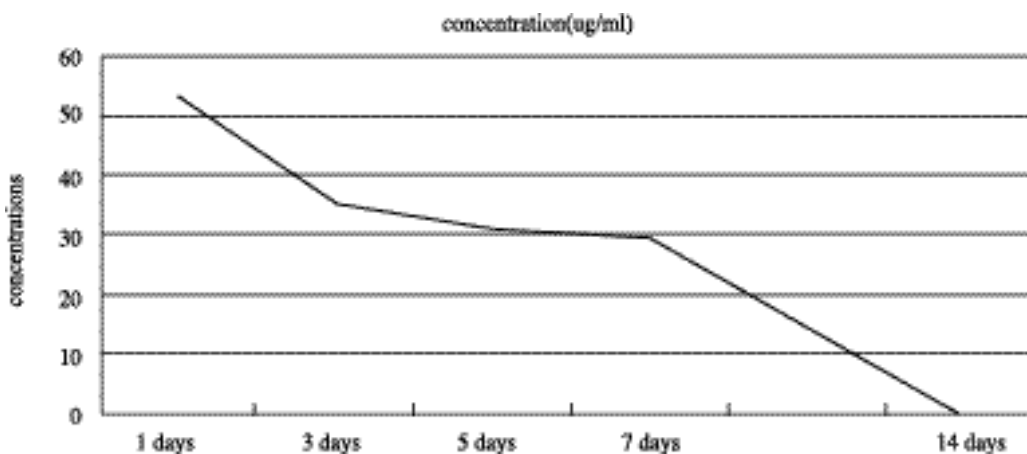


Figure 6. Tetracycline release kinetics. At day 1 Tc was released at $52.50 \pm 14.40 \mu\text{g}/\text{Ml}$. Tc was steadily released at day 3, 5, 7 at $35.00 \pm 13.42 \mu\text{g}/\text{Ml}$, $30.83 \pm 14.63 \mu\text{g}/\text{Ml}$, $29.17 \pm 13.20 \mu\text{g}/\text{Ml}$ each. On the 14th day, no Tc was detected.

till the 4th week. GI of the Tc - unloaded group (Group II) or of the control group (Group III) was slightly higher than that of the Group I throughout the study. But no statistical difference was seen

between the groups (Table 1, Figure 1). Plaque index of the Group I and Group III decreased with time. But in the Tc - unloaded membrane group, plaque index increased. In Group I, the PI scores at 4th

week was significantly less than that at the baseline ($p < 0.05$), in Group II, this was higher at 1st week and maintained at this level at 2, 4 weeks but these scores were slightly higher than that of the Group I or than that of the Group III. In Group III, PI at 1st week did not differ from the baseline but showed a tendency to be decrease at 2 and 4 weeks (Table 2, Figure 2). The volume of gingival crevicular fluid measured with Periotron was similar in all the groups. The volume measured at the first week was higher than that of the baseline but this decreased and at the 4th week, the measured volume was similar to that of the baseline (Table 3, Figure 3).

3. Microbiological Assay

Total anaerobic and aerobic colony forming units of each group is shown in tables 4, 5 and figures 4, 5. CFUs of anaerobic and aerobic bacteria were expressed as log10 of the value.

(1) Anaerobic Bacteria

In the Group I, post - operative number of total colony forming units was decreased at 1 week and rebounded at 2, 4 weeks slightly. In the group II and III, CFU were slowly decreased throughout the study (Table 4, Figure 4). In the view of time point, there was significant differences group I and group II, III at 1 week. Also, there were significant differences between group II and group I, III at 2, 4 weeks ($p < 0.001$).

(2) Aerobic Bacteria

In the group I, as the pattern of cfu of anaerobic bacteria post - operative number

of total colony forming units was decreased at 1 week but rebounded nearly baseline level at 2, 4 weeks. In the group II and III, CFU were slowly decreased throughout the study (Table 5, Figure 5). In the view of time point, there was significant differences group I and group II, III at 1 week. But, there were no significant differences among any groups at 2, 4 weeks.

4. Tetracycline Release Kinetics

To test the release kinetics of loaded tetracycline from the membrane, the diameter of the inhibition zone formed in the nutrient agar plate was measured and the concentrations of released tetracycline were calculated from the standard curve prepared. This was measured at a total of 6 sites and the average concentration was calculated. On the first day, the measured concentration was $52.50 \pm 14.40 \mu\text{g}/\text{Ml}$ which is quite high and on the 3rd, 5th and the 7th day, this was released consistently at $35.00 \pm 13.42 \mu\text{g}/\text{Ml}$, $30.83 \pm 14.63 \mu\text{g}/\text{Ml}$, $29.17 \pm 13.20 \mu\text{g}/\text{Ml}$ each (Table 6, Figure 6). But on the 14th day, Tc was not detected.

IV. Discussion

This study was done to evaluate whether Tc - loaded membranes can inhibit contamination and bacterial colonization of the membrane, and infection of the surgical site in initial healing period. Tetracycline, a wide - spectrum antibiotic was loaded in a biodegradable membrane and the following was investigated 1) Whether Tc - loaded membrane could decrease clinical signs of

inflammation and the number of bacteria found in a experimental periodontitis site in a beagle dog 2) the release kinetics of Tetracycline.

Membranes used in this study were not seen to be cytotoxic in a previous study using the polymer used in this membrane⁴³). This membrane started to be degraded 4 - 6 weeks after placement in the subgingival tissue of rat without any adverse tissue reaction⁵⁰).

The decrease in clinical gingival inflammation and plaque accumulation in the Tc-loaded membrane group was greater than that of the Tc-unloaded membrane group, but this difference was not statistically significant. In the Tc-loaded membrane group, GI and PI tended to be decreased from post-op week 1 to week 4. But, GCF volume measured by Periotron increased at 1 week after surgery in all three groups and decreased to same level as baseline and was similar between all three groups throughout the test period. The total colony forming units(CFU) of anaerobic bacteria in the Tc-loaded membrane group was less than that of the Tc-unloaded membrane group at all time points. Though rebounded at 2week, the cfus of both anaerobe and aerobe of Tc-loaded membrane group were significantly decreased at 1week compared to not only baseline but also the other groups. This antibacterial effect may be helpful initial healing of the tissue regenerative process.

In this study, the concentrations of tetracycline released from the membrane was indirectly measured by sampling Tc released in the gingival sulcus with peri-

opapers⁴⁷⁻⁴⁹). The level of Tc released from the membrane was the highest at day 1 at $52.50 \pm 14.40 \mu\text{g}/\text{Ml}$, and on day 3, 5, 7, was steadily released at the concentrations of $35.00 \pm 13.42 \mu\text{g}/\text{Ml}$, $30.83 \pm 14.63 \mu\text{g}/\text{Ml}$, $29.17 \pm 13.20 \mu\text{g}/\text{Ml}$. But, Tc was not observed at day 14. Kim et al⁵¹ have reported in an in vivo study that Tc was released from a 10% Tc-loaded membrane at the concentration of $52 \mu\text{g}/\text{Ml}$ at day 1 and was steadily released thereafter for 4 weeks. But in that study, the release kinetics of the Tc was seen by removal of Tc at various time points after placement of the membrane in the subcutaneous tissue in the rat. Markman et al tested a cellulose membrane containing Tc to investigate its release kinetics but this experiment was also under closed conditions. In that experiment, slow release of Tc was seen at concentration of $218 \mu\text{g}/\text{Ml}$ at day 1 and this release was continued till day 12 at $20.8 \mu\text{g}/\text{Ml}$ ⁵²). In this experiment, Tc-loaded membrane was placed in periodontitis-alveolar bone defect and gingivitis-induced site in beagle dog, and released Tc in the sulcus was measured. Most microorganisms found in the gingival sulcus were susceptible to $8 \mu\text{g}/\text{Ml}$ of Tc²⁷. Though Tc was not detected in the sulcus at day 14, it was slowly released to the sulcus at levels above MIC till day 7.

Relatively many GTR barriers are exposed to the oral environment. Some studies have reported that in 70% of GTR cases, membranes were exposed and bacteria found on the surface of the exposed membranes^{10,19}). Infection of the surgical site and the membrane can occur between

the gingival flap and the tooth surface. Also when the membrane is exposed, bacterial infection of the membrane is enhanced and as the apical migration of the epithelium occurs, a pocket - like structure is formed external to the membrane. Bacterial colonies have been observed with light microscopes on both the external and the internal surface of the membrane⁴⁾. Mombelli et al has reported that putative periodontal pathogens were cultured from retrieved GTR barriers. Gram - negative, anaerobic rods made up 31% of the total organisms from all samples⁵³⁾. It is not clear to what extent bacterial colonization of GTR materials compromise success of therapy, but it can be safely assumed that microbial colonization causes complications. Some have reported that attachment of bacteria on the removed barrier membranes are related to limited clinical attachment gain^{10,16,54,55)}.

Systemic antibiotics have been used after GTR therapy to decrease the incidence of infection of the surgical site or membrane. Demolon et al reported that when microbiological assay with DNA probes were done on bacteria sampled with paper points, total number of bacteria increased when time passed regardless of use of post - operative systemic antibiotics²¹⁾. It was concluded that when a membrane is placed, the number of bacteria is increased as time passes whether systemic antibiotics are used or not^{21,56)}. Local application of chlorhexidine is necessary to inhibit infection of the membrane or surgical site, but this is limited to post - operative 1st week when it is difficult for the patient to maintain oral

hygiene. In this study, almost no oral hygiene measures were performed for 2 weeks after surgery. After this period, limited oral hygiene measures were taken 2 - 3 times a week with chlorhexidine. This differs from usual human studies. When membranes are placed in human subjects, postoperative chlorhexidine gargling is prescribed and professional oral prophylaxis is done to inhibit contamination of the membrane or infection of the surgical site. As mentioned previously, the results of this study show that though Tc - loaded membrane is more effective than Tc - non - loaded membrane, as membranes tend to be infected by bacteria, the number of colony forming units found in the Tc - loaded membrane group, was greater than that found in the control group. Meticulous plaque control during surgery and post - operatively or the use of wide - spectrum antibiotics cannot completely inhibit bacterial contamination of the membrane or infection of the surgical site. Aseptic technique is necessary to offer better conditions for regenerative therapy. Local application of antibiotics directly on the membrane may be more effective⁵⁷⁾.

In this study, to prevent crossover effect of Tc, split mouth design was not used. Because, it is possible for Tc released from one site in the oral cavity to influence other sites. It has been shown that following placement of tetracycline fibers, the drug is transiently detected in the serum or saliva⁵⁸⁾, which may effect the microbiological response. Tc was shown to be slowly released at concentrations higher than MIC and inhibit gingival inflammation and growth

of bacteria in the oral cavity. Thus, the use of Tc - loaded membranes can result in improved clinical outcome from adjunctive antiinflammatory and antimicrobial effect of tetracycline on guided tissue regeneration using barrier membrane.

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VI. References

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