Antimicrobial Activities of Root Surfaces Treated with Tetracycline-Containing Gel and a Mixture of Tetracycline and Citric Acid-Containing Gel : *In Vitro* study

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I . Introduction

The most important treatment modality of periodontal disease is the removal of bacterial plaque. This is generally accomplished by subgingival instrumentation, i.e., deep scaling and root planing and patient oral hygiene instruction[1,2]. The knowledge that bacterial plaque is the main etiologic factor in chronic inflammatory periodontal disease, and specific organisms are responsible for specific disease process[3-5] provides a rationale for the use of antibiotics as an adjunctive therapy to scaling and root planing. Thus antimicrobial therapy has been directed at specific bacteria associated with clinically diseased sites to help augment the mechanical treatment aimed at the removal of subgingival calculus and toxins, especially individuals who failed to respond adequately to mechanical therapy[6,7].

As systemic use of antibiotics may cause several adverse effects[8], recently the local administration of antibiotics has received considerable attention[9-14]. For example, tetracycline HCl and chlorhexidine digluconate, as ir-

rigation solution or incorporated in sustained release system for subgingival delivery, have been evaluated widely. When applied locally, these drugs have proven effective in reducing suspected periodontal pathogens and in improving clinical health.

An improtant prerequisite for an antimicrobial drug as a plaque-inhibiting agent is substantivity. Tetracycline and chlorhexidine seem to be widely used as supplements to periodontal therapy. Both drugs have broad antimicrobial activity and substantivity. Chlorhexidine digluconate is substantive when enamel or dentin are exposed[15]. But little is known of its interactions in a subgingival environment.

In vitro studies have shown substantivity of tetracycline to tooth surface including dentin[16-21]. Tetracycline was the drug of choice because of its antimicrobial activity against the subgingival microflora[22-25], as well as its ability towards improving clinical results. It has been shown *in vitro* to remove the smear layer, cause surface demineralization and adsorb to dentin[18,26-29]. Following adsorption tetracycline releases while still maintaining bac-

teriostatic concentration for most known periodontopathogens[30]. Stabholz et al[21]. demonstrated that tetracycline may be retained in the subgingival environment to slowly release to the gingival crevicular fluid in clinically meaningful concentration following an irrigation procedure. Non-antimicrobial benefits of tetracycline may include ability to inhibit collagenolytic enzymes[31-34].

Therapeutic drug concentrations have also been demonstrated for tetracycline and chlorhexidine in periodontal pockets following insertion of slow-release devices[11,13,14,35]. Methods for local delivery of tetracycline have been investigated and include an acrylic resin delivery system resorbable collagen film[36], and a resorbable polymer microincapsulation system. However, most present drug delivery system seem to require both professional placement and removal. Moreover, the delivery devices may become unintentionally and prematruely dislodged from their site of action.

Numerous previous studies demonstrated that solution type of tetracycline HCl exhibited marked substantivity on dental hard tissue[16-21, 37,38], and clinical and microbiological effects on periodontitis[39,40]. For clinical use, the solution should be made freshly because of its unstability. Because preparing the solution is time-consuming procedure, it makes clinicians avoid the use of tetracycline solutions. To overcome that problems, we developed gel type tetracycline HCl and a mixture of tetracycline-citric acid gel for subgingival delivery. Jeong et al[41]. had already reported its clinical and microbiological effectiveness.

Thus we designed this study for the purpose of assessing *in vitro* substantivity of newly developed tetracycline-containing gel and a mixture of tetracycline citric acid-containing gel, and compare to those of chlorhexidine digluconate.

I. Materials and Methods

Subjects teeth

18 extracted single rooted teeth (8 upper and 10 lower anteriors) from patients with advanced periodontal disease (probing depth>6mm) in 6 patients were involved in this study. All teeth were scheduled for extraction as a part of the treatment plan for these patients.

Proir to entry to this study, each patient or tooth satisfied the following inclusion criteria : 1) no history of systemic disease : 2) no history of antibiotic therapy in the previous 3 month : 3) no caries of individual tooth.

Experimental design

After extraction teeth were scaled and root planed in an attempt to remove the root cementum and autoclaved. Teeth were sealed with nail enamel in their apical aspects to exclude communication to the root canal.

They were then randomly divided into 4 treatments groups : group 1) ; 4 teeth were immersed in tetracycline HCl(50mg/ml) solution for 5 minutes at room temperature ,group 2) ; 4 teeth were immersed in tetracycline gel (5%) for 5 minutes , group 3) ; 4 teeth were immersed in a mixture of tetracycline and citric acid gel, group 4) ; 4 teeth were immersed in 0.1% chlorhexidine digluconate. And 2 roots immersed in 0.9 % sterile saline served as controls.

Preperation of drugs

Tetracycline HCl solutions were freshly prepared in distilled water from capsule tetracycline HCl powder(Chong-Kun Dang, Seoul, Korea). For 50mg/ml solution, the drug was dissolved at 53℃ in a water bath. The solutions were filtered with 0.22μm pore sized filter(Syrfil-MF(25mm, 0.22μm, Max press 75

PSI), Nuclepore) to remove capsule filler particles.

The constituents of tetracycline gel were poloxamer(gelating agent, Junsei Chemical Co., Ltd., Seoul Korea), tetracycline HCl(Sigma Chemical Co., St.Louis, MO.) and distilled water. The composition of mixture gel was ethanol, citric acid(Duksan Pharmaceutical Co., Ltd., Seoul, Korea), carbopol(gelating agent, Junsei Chemical Co., Ltd., Seoul Korea), tetracycline HCl and distilled water. The concentration of tetracycline of both gels was 5%, and the concentration of citric acid was 33%. 5% tetracycline gel corresponds to 50 mg/ml tetracycline solution and this preparation had basis on the *in vitro* previous studies [18,20,21]. Commercially prepared 0.1% chlorhexidine digluconate(Hexamedin, Pukwang Pharmaceutical Co., Ahnsan, Korea) and 0.9% sterile saline(Choong-wae Pharmaceutical Co., Korea) were used.

Drug exposure and storage

Each tooth was immersed in 5ml of the respective drug so that the drug completely covered the root for 5 minutes and was vortexed in 10ml of sterile saline for 30s to remove nonadsorbed drug. Immediately following drug exposure, each root was transferred into a tube containing 1 ml of sterile tris buffered saline (TBS, pH=7.4) as a desorption media and was then incubated at room temperature for 22 days. At 24-h intervals, the total volume of TBS was removed and replaced with 1ml of fresh TBS. Removed desorption media transferred to a sterile vial and stored at -70°C. This procedure was repeated every 24-h throughout the 22-day desorption period.

Microbiologic assays

A microtiter assay was used to evaluate antimicrobial activity desorbed from the teeth into incubation solutions. Frozen TBS-aliquots from 22 time intervals for the 18 teeth, a total of 396 samples, were thawed. As an indicator organism Porphyromonas gingivalis W50 was used. This bacterial strain was selected because of its availability in our laboratory and because it is considered as a major periodontopathic microorganism[5].

A stock cultures of P.gingivalis were transferred to Brain Heart Infusion (BHI) broth supplemented and were incubated at 37°C for 48 hours in an anaerobic condition of 85% N2, 5% H_2 and 10% CO_2. 70μl of solution from each thawed sample and 30μl of the bacterial suspension in BHI were dispersed onto 96-well tissue culture plates(Nunclon, Nunc, Denmark) and incubated at 37°C for 40 hours in an anaerobic chamber. Negative controls consisting of sterile media and positive controls for bacterial growth were added to each plate. Following incubation, bacterial growth was assayed by reading the optical density (OD) at 550 nm, using an ELISA plate reader (Thermomax, Molecular device corp., California, USA.). All assays were performed in duplicate.

Data analysis

Statistical analysis was done using a SPSS PC+ computer program. To define the significant interaction between time and changes in OD, the data was analyzed using correlation and regression analysis. One way analysis of variance(ANOVA) was performed to compare the 4 treatments and controls at each time point with optical density as the dependent variable and treatment as independent variable. To identify which means were significantly different from controls, the Scheff test was used. A value of p<0.01 was considered signi-

ficant.

III. Results

Regression coefficients and correlations for the study conditions from day 1 through 22 are presented in table 1. Regression coefficient for the 2 tetracycline-containing gels were very similar, and that of tetracycline solutions exhibited a steeper slope. While the regression coefficient of chlorhexidine digluconate were nealy zero. Regarding correlations, tetracycline solution showed the highest value.

Table 2 represents the ANOVA for OD's following the 4 treatments at each time point.

Table 1. Regression coefficient(Reg Coeff.),Probabilities(Prob.) and Correlations(Corr.) for the Optical Density readings for the different treatments.

Treatment	TCS	TCG	TC-CAG	CHX
Reg. Coeff.	0.021	0.014	0.015	0.000
Prob.	0.000	0.000	0.000	0.000
Corr.	0.884	0.601	0.735	0.542

SAL = Saline
CHX = Chlorhexidine digluconate
TCS = Tetracycline HCl solution
TCG = Tetracycline-containing gel
TC-CAG = Tetracycline and Citric acid-containing gel

Table 2. Mean Optical Densities of Each Treatment Group at Different Time Point

Day TX.	1	3	7	11	17	22
SAL	0.97	0.97	1.00	1.03	1.02	1.05
	± 0.01	± 0.02	± 0.07	± 0.04	± 0.01	± 0.06
CHX	0.63*	0.93	0.95	0.98	1.00	1.01
	± 0.13	± 0.03	± 0.04	± 0.03	± 0.05	± 0.03
TCS	0.50*	0.53*	0.55*	0.63*	0.79*	0.93
	± 0.08	± 0.06	± 0.08	± 0.06	± 0.06	± 0.04
TCG	0.59*	0.66*	0.79	0.79	0.86	0.95
	± 0.04	± 0.09	± 0.13	± 0.14	± 0.12	± 0.10
TC-CAG	0.61*	0.80*	0.89	0.92	0.98	1.00
	± 0.13	± 0.09	± 0.08	± 0.09	± 0.04	± 0.04

SAL = Saline
CHX = Chlorhexidine digluconate
TCS = Tetracycline HCl solution
TCG = Tetracycline-containing gel
TC-CAG = Tetracycline and Citric acid-containing gel
* : significantly different from Saline-Treated group ($p < 0.01$)

The effect of each treatment was compared to the saline control. The one way ANOVA revealed significant differences between the ODs of tetracycline HCl 50mg/ml and those of the saline control group from desorption day 1 through 17.

The ODs of 5% tetracycline gel were significantly different from day 1 through day 4, and those of a mixture of tetracycline and citric acid gel were significantly different through day 3. This difference was statistically significant from day 1 through day 4 of tetracycline-containing gel, and day 3 of a mixture of tetracycline and citric acid-containing gel respectively. After the period, the mean ODs of tetracycline gel were still lower than those of a mixture of tetracycline and citric acid gel, but that difference was not statistically significant ($p<0.01$). Whereas the chlorhexidine digluconate solutions showed significant antimicrobial activity during the first 24-h only.

The ODs for desorption fluids for each of the 4 drug treatments over the 22-day period were presented as graphs in fig. 1-5. For comparison purposes the OD for the positive controls ranged between 0.90 to 1.02, and for the negative controls ranged between 0.19 to 0.27. Teeth immersed in tetracycline HCl 50 mg/ml solution maintains its antimicrobial activity for the longest time followed by teeth exposed in tetracycline-containing gel.

The ODs of desorption fluids from teeth exposed to chlorhexidine digluconate were not different from those from teeth immersed in sterile saline day 2 through 22 (Fig.1). The ODs of controls(immersed in saline) slightly fluctuated around a value of 0.99 throughout the study period.

The ODs of the 2 tetracycline-containing gels were gradually increased for the early period(around day 5), and then exhibited elevated OD readings (Fig.3, Fig.4). Viewing Fig.
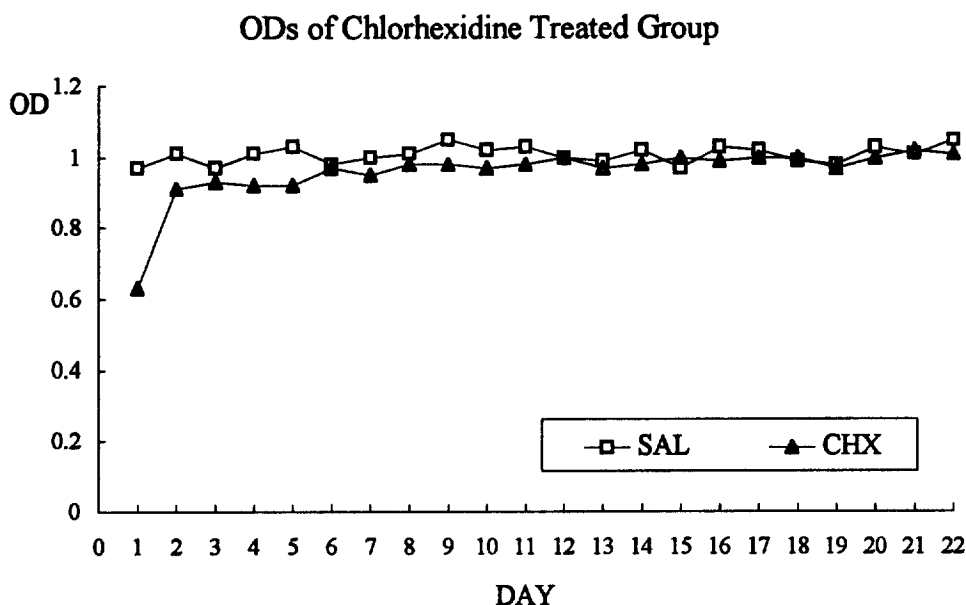
ODs of Chlorhexidine Treated Group

Fig.1. Optical density readings(OD) for 22-day period of media of teeth immersedin chlorhexidine digluconate(CHX) and saline(SAL).

1 we note that OD values of saline and chlorhexidine digluconate gave essentially a horizontal line over time, while tetracycline HCl showed an increase over time until the OD levels reach those of control. The two types of gels followed very simillar pattern. And at each time point, the mean ODs of 5% tetracycline gel were lower than those of a mixture of tetracycline and citric acid-containing gel.
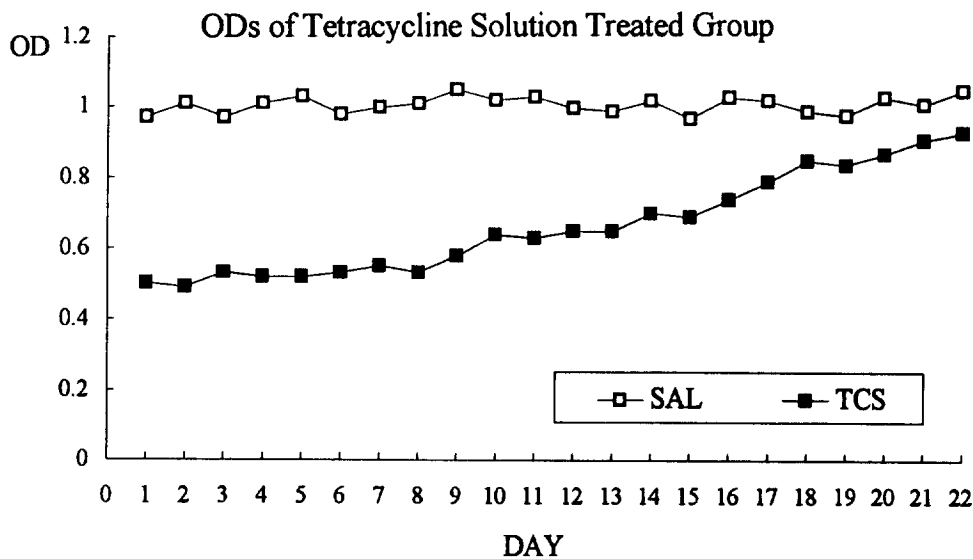

Fig.2. Optical density readings(OD) for 22-day period of media of teeth immersed in tetracycline HCl solution(TCS) and saline(SAL).
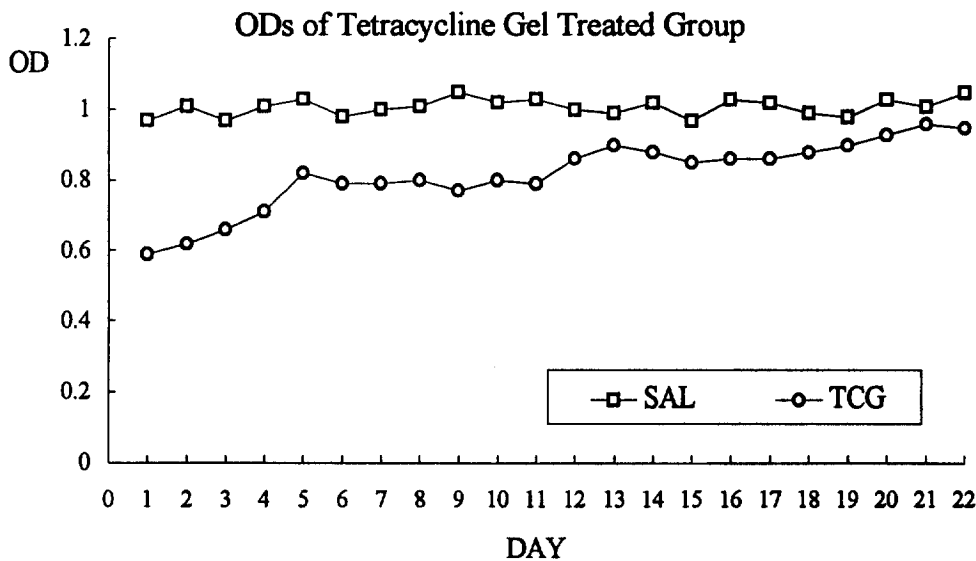

Fig.3. Optical density readings(OD) for 22-day period of media of teethimmersed in tetracycline-containing gel(TCG) and saline(SAL).

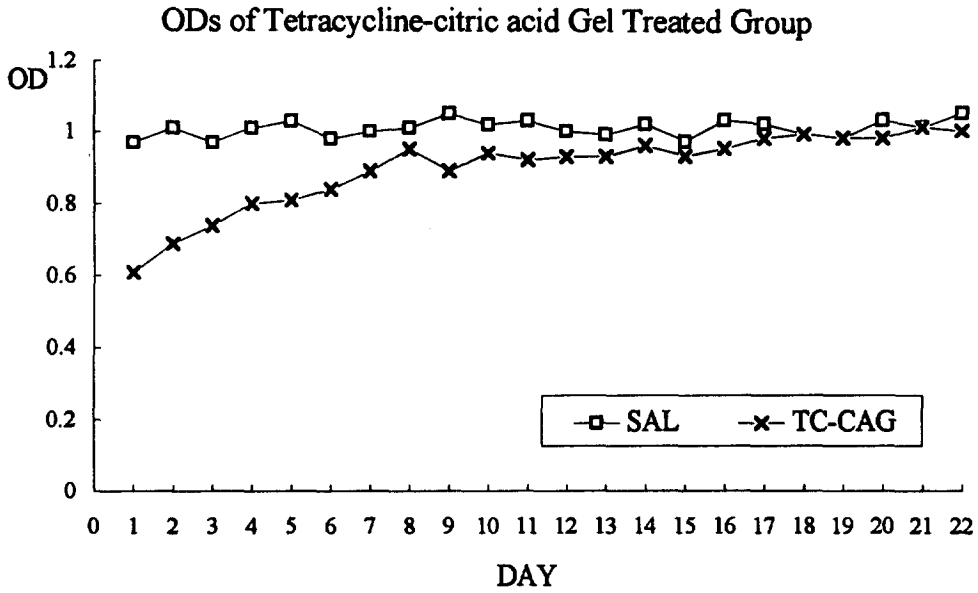377

ODs of Tetracycline-citric acid Gel Treated Group

Fig.4. Optical density readings(OD) for 22-day period of media of teethimmersed in tetracycline and citric acid-containing gel(TC-CAG) and saline(SAL).
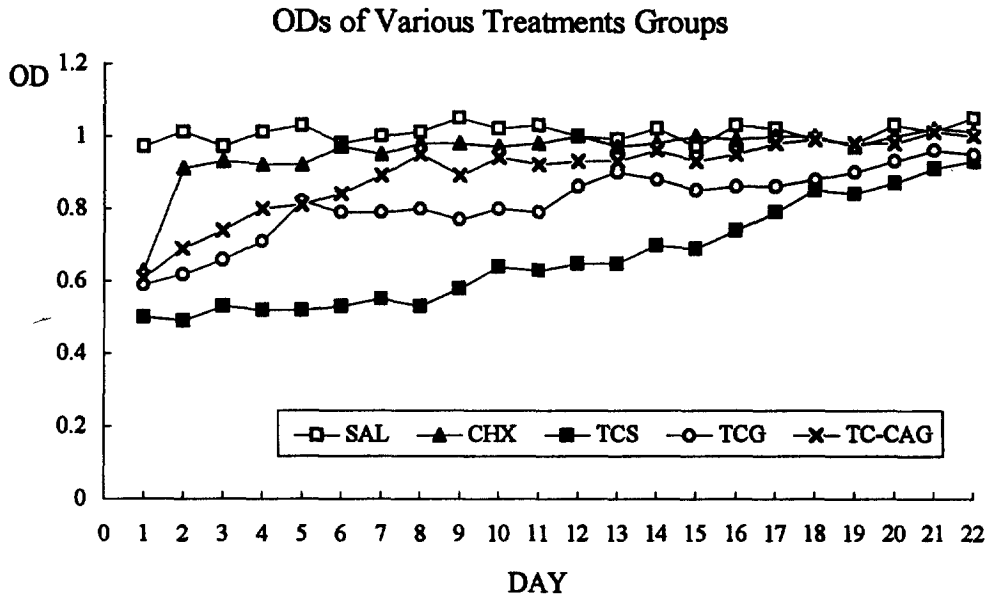
ODs of Various Treatments Groups

Fig.5. Optical density readings(OD) for 22-day period of media of teethimmersed in various drugs treatment.

CHX=Chlorhexidine digluconate
TCS=Tetracycline HCl solution
TCG=Tetracycline-containing gel
TC-CAG=Tetracycline and Citric acid-containing gel

Ⅳ. Discussion

This study based on previous work[41] describing the clinical and microbiological effects of subgingival application of two tetracycline-containing gels was designed to compare the substantivity of two tetracycline-containing gels with its solution type. The results of this study demonstrated that solution was the most substantive form of the drug, and two tetracycline-containing gels showed similar antimicrobial activities.

Compared to saline treated group, 50mg/ml tetracycline HCl solution treated group exhibited significantly different optical densities throughout 17-day desorption period. The ability of tetracycline to adsorb to tooth surface may have contributed to the clinical and microbiological effectiveness of this drug. Some authors have presented that root surfaces treated with aqueous tetracycline solution exhibited slow release of the drug in vivo. Puchalsky et al[37]. reported that tetracycline was detected in GCF for 7 days following a single 5-min. irrigation of periodontal pockets with a 100mg/ml aqueous solution. Recently Stabholtz et al[21]. demonstrated root surface irrigated with 50mg/ml tetracycline solution for 5-min. exhibited antibacterial properties for 16 days longer than saline treated group.

The substantivity of 5% tetracycline-containing gel was significantly longer than control group from day 1 through day 4, and that of a mixture of tetracycline-citric acid gel was significantly different through day 3. Considering that the concentrations of 3 types of tetracycline HCl were same, the component of gels -poloxamer or carbopol- may inhibited the adsorption of the drug to the root surface. Although the differences between the ODs of both gels were not statistically meaningful, generally the mean ODs of tetracycline gel were lower than those of a mixture gel.

Placement of tetracycline directly into the gingival sulcus has been explored as a means of bypassing systemic complications and delivery the drug only at the diseased site. This has been accomplished with subgingival irrigation or insertion of drug-loaded devices. Compared to non-surgical periodontal therapy, irrigation with tetracycline has generally demonstrated little additional effect[42]. Furthermore conventional use of aqueous solution of tetracycline would have caused several problems, for example difficulty of long-term storage and localization at one site to prevent spillover. Therefore we disigned hydrophillic gels as root conditioning and antimicrobial agent for local delivery. We expected 2 tetracycline-containing gels to show substantivity similar to those of tetracycline solution. Besides we expected the higher substantivity of a mixture of tetracycline and citric acid-containing gel. But according to the results tetracycline solution showed the longest antimicrobial activity, followed by 2 tetracycline-containing gels.

Similar to other *in vitro* and in vivo reports[20,21], this study showed that the antimicrobial activity of chlorhexidine digluconate was maintained during the first 24-h only. But researches using chlorhexidine digluconate as subgingival irrigation agent showed its marked clinical and microbiological effects[12,43-45]. Thus we could say that substantivity alone is not a critical requisite as oral antimicrobial agent for periodontal disease.

Local delivery systems for chemotherapy of the periodontal pockets have attracted a wide interest in recent years. According to Goodson et al[14]., the total patient dose following local tetracycline delivery can be significantly reduced when compared to systemic administration. It may be especially indicated

for individual unresponsive sites and in the maintenance of treated periodontal pockets.

Concerns over the development of hypersensitivity with topical applications of tetracycline have been previously voiced[46]. Another unresolved question is the development of resistant bacteria following local applications of antimicrobials. Bacterial resistance following systemic administration of tetracycline has been observed by a number of investigators[8,47–49]. However, Goodson et al[50]. recently reported that locally delivered tetracycline did not promote the development of tetracycline resistant gram negative rods or induce polyantibiotic resistance. Even so, further study using repeated applications over prolonged periods of time is indicated.

One disadvantage with local antibiotic therapy is difficulty of applying the agents to deeper parts of the periodontal pocket. Local therapy may also be relatively time-consuming if many periodontal sites have to be treated. Some authors developed tetracycline-containing pastes which can be easily delivered subgingivally in a petrolatum base[51–53]. They reported biologically effective tetracycline was released into GCF for at least 3 days after subgingival delivery of tetracycline in a white pertolatum carrier. But they found it cannot replace scaling and root planing therapy. We designed a mixture of tetracycline and citric acid gel to improve the properties by increasing its root conditioning effect. Based on the previous clinical study of Jeong et al[41]., we expected the more substantivity of the mixture gel than gel which doesn't contain citric acid. But the result showed no additive effect of mixture gel on antimicrobial substantivity. Although substantivity of both gels were much lower than solution, the result of this study could suggest the practical use of the gels on the basis of its antimicrobial and non-antimic-robial activities and substantivity.

Because the extent of tetracycline adsorption to dentin was not determined in the present investigation, further study is needed to differentiate the retention and release dynamics of 2 tetracycline-containg gels from the substantivity of tetracycline itself.

V. Conclusions

The purpose of this study was to evaluate the substantivity of experimentally developed gel type tetracycline HCl and a mixture of tetracycline-citric acid gel, and compare to those of solution type tetracycline HCl. Within limitation, we aquired the following conclusions.

1. 50mg/ml tetracycline HCl solution exhibited the longest antimicrobial activity. Compared to saline treated group, it showed significant difference throughout 17-day desorption period.

2. The ODs of 5% tetracycline gel were significantly different from day 1 through day 4, and those of a mixture of tetracycline-citric acid gel were significantly different through day 3.

3. Chlorhexidine digluconate solutions showed significant antimicrobial activity during the first 24-h only.

4. Throughout the period the mean ODs of tetracycline gel were generally lower than those of a mixture of tetracycline and citric acid gel, but that difference was not statistically significant.

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국문초록

테트라사이클린 및 테트라사이클린-구연산 혼합젤로 처리한 치근면의 항미생물 활성 변화에 관한 연구

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본 연구의 목적은 비외과적 치주치료시 부가적으로 사용하기 위해 실험적으로 개발한 젤 형태의 테트라사이클린 및 테트라사이클린-구연산 혼합젤의 치근면에 대한 시간에 따른 활성도를 측정하고, 이를 용액 형태의 테트라사이클린 제제 또는 클로르헥시딘 들과 비교하는 것이다. 6명의 환자로 부터 18개의 발치된 치아를 실험대상으로 하였으며, 치아는 발치한 즉시 치석제거술과 치근활택술을 시행한 후 각 각 4개씩 4군으로 나누어 다음과 같은 처치를 하였다 ; 1) 0.1% 클로르헥시딘 용액에 5분간 침전 ; 2) 50mg/ml의 테트라사이클린 용액에 5분간 침전 ; 3) 5% 테트라사이클린 젤에 5분간 처리 ; 4) 테트라사이클린-구연산 혼합젤로 5분간 처치 ; 5) 그리고 2개의 치아는 대조군으로서 멸균된 생리식염수에 5분간 처리하였다. 침전후 치아는 1ml의 tris-buffered saline이 담긴 용기에 옮겨 24시간 간격으로 탈착된 TBS용액을 교체하면서 실온에서 22일간 보관하였다. Porphyromonas gingivalis를 indicator organism으로 하여 microtiter assay를 이용하여 흡광도를 측정함으로써 제거된 용액의 항미생물 활성을 측정하였다.

1. 50mg/ml의 테트라사이클린 수용액에 침전되었던 군은 생리식염수로 처리한 군에 비하여 17일간 클로르헥시딘으로 처리한 군에 비하여는 16일간 항미생물 활성에 있어서 유의성 있는 차이를 보였다.
2. 테트라사이클린 젤과 테트라사이클린-구연산 혼합젤로 처리한 군은 대조군에 비하여 각 각 4일과 3일 까지 활성을 보였다.
3. 0.1% 클로르헥시딘 용액으로 처리한 군은 생리식염수로 처치한 군에 비하여 24시간 밖에 활성을 나타내지 못했다.
4. 전반적으로 테트라사이클린-구연산 혼합젤로 처리한 군에 비하여 테트라사이클린 젤로 처리한 군의 활성이 높았으나 유의성 있는 차이를 보이지는 않았다.

주요어 : 테트라사이클린, 테트라사이클린-구연산, 젤, 항미생물 활성