Inhibition of Plaque Formation on the Titanium Surface by Anti - bacterial Varnish

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I. Introduction

For the dental implant to be successful, the implant fixture should be placed in jaw bone with optimal quantity and quality under good surgical technique, and the prosthetic superstructure should be constructed under the principle of optimum functional occlu - sion and passive fitness. Besides, bacterial plaque should be controlled to prevent peri - implant tissue inflammation and sub - sequent bone loss. Bacterial contamination on implant surface starts as soon as any part of dental implant system are commu - nicated with oral environment following the second stage surgery in 2 - stage implant system or implant placement in 1 - stage implant system[1]. Especially, it was shown there is inflammatory infiltrates in peri - implant tissue around the implant fixture - abutment interface[2]. The reduction in plaque build - up is of major importance to prevent peri - implant mucositis around oral implants[3]. Therefore, bacterial contamina - tion upon implant placement and plaque formation around implant superstructure should be prevented from the stage of implant surgery.

In preventing and treating dental infection, the substantivity of the anti - microbial agent is of utmost importance since the agent needs a certain amount of contact time with microorganisms in order to inhibit or kill them. Substantivity is a measure of the contact time between a substance and a substrate in a given medium. For any degree of substantivity, adherence of sub - stance to substrate should be greater or more prolonged than would be expected with simple mechanical deposition[4].

Chlorhexidine has been known as one of very effective anti - microbial agents in the prevention and treatment of gingivitis and inhibiting recolonization of plaque bacte - ria[5,6]. One reason chlorhexidine exerts a greater anti - plaque effect than other anti - microbials is its remarkable ability to adsorb to tooth and gingival surfaces and then

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releases into the oral cavity over time at therapeutic levels[7]. Chlorhexidine is a synthetic cationic detergent with great bacteriostatic and bactericidal activity against Gram positive and negative microorganisms and against yeast cells, has a great affinity for the cell wall of microorganisms and changes the surface structures. Osmotic equilibrium is lost, and as a consequence, cytoplasmic membrane is extruded, vesicles are formed, and the cytoplasm precipitates[8], which inhibit the repair of the cell wall and the bacteria are no longer able to recover. These cationic molecules attach to anionic compounds such as free sulfates, the carboxyl and phosphate groups of the pellicle, and salivary glycoproteins[7] and will, thereby, reduce the adsorption of proteins to the tooth surface needed for the formation of dental pellicle. Coating salivary bacteria with chlorhexidine molecules also alters the mechanisms of adsorption of bacteria to the tooth. Chlorhexidine molecules bound to salivary proteins will be released in 8 - 12 hours in active form[9,10] and low concentrations of chlorhexidine can still be recovered after 24 hours. This prolonged bacteriostatic effect of chlorhexidine is an important complement to its high initial bactericidal activity[11]. Vehicles for clinical application of chlorhexidine include mouth rinses and gels. Balanyk and Sandham(1985)[12] developed a varnish system from which active chlorhexidine is released more slowly after being applied.

Besides its substantivity as an antimicrobial agent, tetracycline has anti-collagenolytic activity which is important in periodontal tissue degradation. Holen and his collegues[13] treated titanium surface with tri-dodecyl-methyl-ammonium chloride as cationic surfactant and found that the increased amount of tetracycline adsorbed into titanium surface and the attachment of plaque bacteria was reduced on this surface.

This study was aimed to investigate whether chlorhexidine varnish system and tetracycline treatment could be effective in inhibition of plaque accumulation on the surface of titanium dental implant and abutment by evaluating the dynamics of drug desorption from the treated titanium surface and by examining *in vivo* plaque formation on the titanium surface.

II. Materials and Methods

1. *In vitro* study

Table 1. Grouping according to the treatment

treatment / group\	passivation (28% HNO_3 x 2h)	TDMAC (5% ethanol)	TC (50 mg/ml pH 8)	Chlorhexidine varnish	
				chlorhexidine acetate	polyurethane
Group TC	-	-	+	-	-
Group PTTC	+	+	+	-	-
Group CV	-	-	-	+	+
Group C	-	-	-	+	-
Group PTCV	+	-	-	+	+
Group PTC	+	+	-	+	-

1) Treatment of titanium film with test agents

Films of pure titanium(Hankook titanium Co. Incheon, Korea) were prepared by cutting into 4 x 4mm rectangle and sonic cleansing, and were grouped according to the type of treatment. For passivation, the films were immersed in 28% nitric acid solution for 2 hours, rinsed three times in distilled water for 5 min, and dried overnight in lamina flow bench. To improve the drug adsorption, they were immersed into 5% tri-dodecyl-methyl-ammonium chloride(TDMAC, Aldrich Chemical Co. MI, USA) solution in ethanol as cationic surfactant for 1 hour and dried in air. These films were sterilized under UV light for 15 min.

Substantivity of tetracycline HCl or chlorhexidine acetate to titanium film was assessed *in vitro*. Thirty six films were assigned to one of 6 treatment groups. For anti-microbial treatment, the pre-treated films were placed into 50 mg/㎖ tetracycline HCl solution, pH 8.0(Demeclocycline HCl, Lot No. DT95100S, Lederle) for 1 min. following pre-treatment assigned and dried in a container protected from light for 1 day. For chlorhexidine treatment, we used the commercial varnish system (Chlorozoin^{R}, Knowell Therapeutic Technologies Inc. Toronto, Canada) which consisted of 10% chlorhexidine acetate solution containing 20% Sumatra benzoin in absolute ethanol as the 1st phase component and poly-urethane protectant as the 2nd phase component. In application procedure, the varnish was applied to the surface with brush, air-dried, and covered with a layer of poly-urethane sealant to maintain it in place for several days.

2) The dynamics of drug desorption

a) The anti-microbial activity of the desorption media

Following treatment, the films were transferred to Eppendorf tubes containing 0.2㎖ desorption media. The tubes were incubated at room temperature for 28 days. Desorption media were replaced at 24 h intervals. Removed media were stored in freezer at -20◦C until being assayed. Artificial saliva(pH 7.4, Arvidson and Johansson[14], as desorption media was composed of the followings: 0.1L of 25 mM K_2HPO_4, 0.1L of 24 mM Na_2HPO_4, 0.1L of 150 mM $KHCO_3$, 0.1L of 100 mM NaCl, 0.1L of 1.5 mM $MgCl_2$, 0.006L of 25 mM citric acid, 0.1L of 15 mM $CaCl_2$. Acidity was adjusted to pH 6.7 and the total volume was made to 1L.

To evaluate the anti-microbial activity released into desorption media and remaining in titanium film after desorption, inhibition of *Bacillus cereus* growth was measured for tetracycline activity and inhibition of *St. mutans* growth for chlorhexidine activity. *B. cereus(*KCTC 1012) and *St. mutans (*ATCC B13) were aerobically grown in 20㎖ Nutrient broth(Difco Laboratory, USA) at 37℃ for 18 hour and the cell suspensions were adjusted to 0.15(Cell No. = 1.5×10^8/㎖) at 550 nm under UV spectrophotometer. Mueller-Hinton broth(Difco Laboratory, USA) containing 1.7% agar(Difco Laboratory, USA) was autoclaved, cooled to 50℃, and mixed with 2㎖ bacterial suspension per 100㎖ of

broth agar solution. The 20㎖ of mixed agar solution was poured into plate of 9㎝ diameter and hardened. After 3~4 wells had been prepared on each agar plate and 10 ㎕ of desorbed media was placed into well, the plates were incubated aerobically at 37℃ for 16 hours and the zone of bacterial inhibition was measured in mm using Vernier caliper.

In each experiment, 10 ㎕ reference solution of the known concentration(1~1000㎍/㎖ for tetracycline HCl and 1~2000㎍/㎖ for chlorhexidine acetate) were used for standard calibration between the concentration of drug solution and the zone of bacterial inhibition, and the anti-microbial activity of the desorbed media was extrapolated.

b) Anti-microbial activity of titanium film after drug desorption

The treated titanium films were extracted in 0.2㎖ of desorption media everyday and removed after 1 day, 1 week, 2, 3 and 4 weeks of desorption, and stored at -20°C until being tested. Anti-microbial activity remaining in titanium films was evaluated by incubating the films placed on the Mueller-Hinton agar plates containing bacterial suspension, according to the procedure used for determining the anti-microbial activity of desorption media.

2. Intra-oral plaque accumulation study

The titanium disks treated with anti-microbial agents were attached to acrylic appliance and placed intra-orally in periodontally healthy volunteers for 1 day, 3 days, 1 week, 2, 3 and 4 weeks. These disks were detached according to the time schedule, processed for SEM, and evaluated for the formation of acquired pellicle and bacterial attachment. For each experiment, non-treated titanium disks were placed as the control in the same mouth.

a) Treatment of titanium disks

Titanium rods with 6mm diameter(Hankook Titanium, Incheon, Korea) were cut into disks of 1.4mm thickness and polished using series of abrasive sand papers(#600, 800, 1000, 1200, 2400 and 4000) and aluminum paste. The disks were ultrasonically cleaned in DDW three times for 5 min each.

For the surface pre-treatment, the methods of application which showed the greatest in drug adsorption and release by *in vitro* experiment were used. For passivation, the disks were placed into 28% nitric acid solution for 2 h, rinsed with DDW three times, and dried in the lamina flow bench. To increase the drug adsorption, they were placed in 5% ID MAC solution in ethanol as cationic surfactant and chloride doping agent for 1 h, air-dried, and placed under UV light for 15 min for sterilization.

For anti-microbial treatment, disks were immersed in 50 mg/㎖ tetracycline HCl solution(pH 8.0) for 1 min and air-dried in light-proof vials for 1 day. Chlorhexidine varnish was applied onto the other disks according to manufacturer's instruction.

b) Subject selection and placement of

Table 2. Scoring system for bacterial plaque formation on titanium surface under SEM

Score	Features
0	Absence of bacteria
1	Bacteria present, as scattered single cells or in small isolated aggregates
2	Bacteria present, but not completely covering the surface
3	Bacteria present in a thick layer completely covering the membrane surface
4	Massive plaque formation including filaments and spirochetes

intra - oral appliance

Male volunteers, ranged from 23 to 26 years of age, were examined periodontally and scaling and prophylaxis was done. Two weeks later, they were re - examined clinically and confirmed as periodontally healthy. For six selected subjects, impression of maxillary dental arch was taken and 2 removable acrylic appliances with clasps were constructed buccally from canine to second molar areas bilaterally. The disks were attached to the buccal flange of premolar and molar area with resin.

No foods were restricted except chewing gum or sticky candy. Every subjects brushed their teeth three times a day for 2~3 min. During tooth - brushing, the intra - oral appliance was placed in "simulated" salivary buffer solution(10 mM PBS, pH 7.3, containing 50 mM NaCl). All subjects were instructed not to use any form of therapeutic mouth rinses and not to remove the appliance except when tooth - brushing. After being placed intra - orally, the appliance was monitored and disks were detached at 2 h, 1 and 3 days, 1, 2 and 3 weeks of exposure, and processed for SEM.

c) Scanning electron microscopy

Detached titanium disks were placed in primary fixing solution(2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.4) for 2 h, rinsed with carbonate buffer, and immersed in osmium tetroxide for 2 h as secondary fixation, rinsed and dehydrated with graded aquous ethanol solution. They were placed in 1:1 and 3:1 mixing solution of isoamyl acetate and ethanol solution, immersed in isoamyl acetate for 24 h, and then dried to critical point. Specimens were coated with gold palladium to the thickness of 20~30 nm and observed under SEM(JSM - 5400, JEOL, Japan) for pellicle formation, bacterial attachment and proliferation, and the amount and the extent of plaque formation. The severity of bacterial attachment and proliferation was scored according to the scoring system modified from the system by Simion et al.[15](Table 2).

III. Results

1. I*n vitro* drug desorption dynamics and residual anti - microbial activity of treated films

The drug concentrations released into desorption media differed among the drugs and the treatment methods. Titanium films immersed in 50 mg/mℓ tetracycline HCl released the effective anti - microbial activity in successive desorption media for

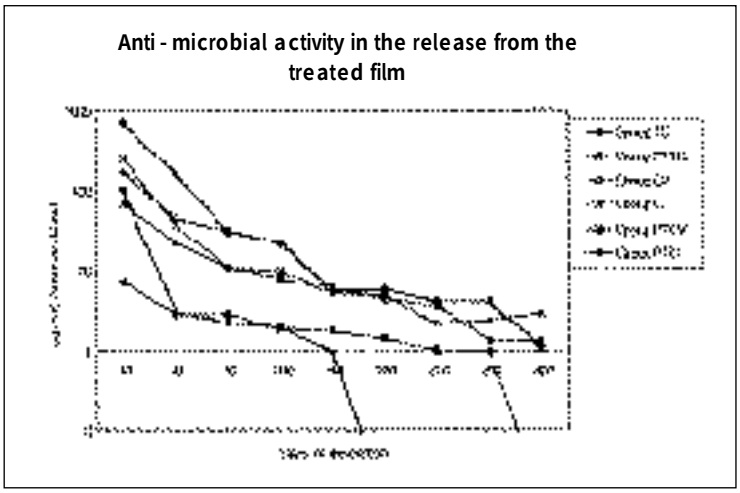

Figure 1. Anti - microbial activity in the desorption media released from the treated film according to the treatment methods.

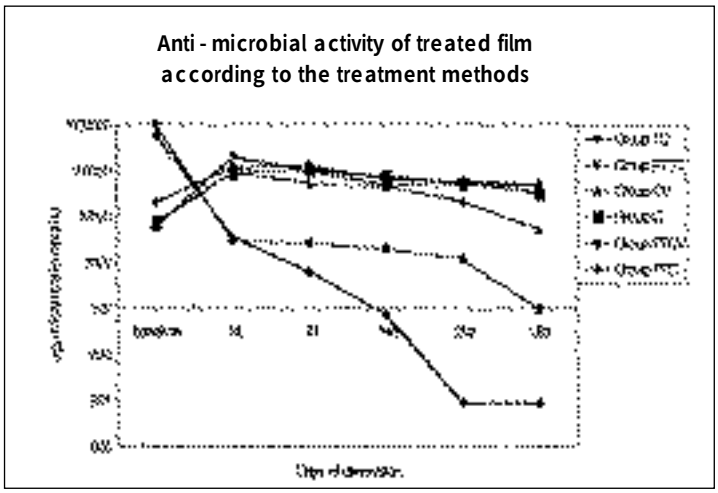

Figure 2. Anti - microbial activity of the treated film after desorption according to the treatment

10~18 days depending on the type of pre - vious treatment. The specimens treated with tetracycline solution only released the drug in the highest concentration during the first day of immersion, thereafter the anti - microbial activity decreased rapidly to 2μg/㎖ in 10 days and to undetectable level after 2 weeks of desorption, whereas the speci - mens treated with tetracycline HCl after cationic surfactant/chloride doping agent showed persistent release of the drug in the concentration above the level of 1μg/㎖ in 18 days.

In the films subjected to chlorhexidine acetate, there was difference in the anti - microbial activity among the specimens according to the surface protectant treat - ment and pre - treatment methods. The

specimen without surface protectant after cationic surfactant treatment released the active drug in higher concentration at 3 day of desorption and thereafter decreased to 21μg/mℓ after 10 days of desorption. The specimens treated with chlorhexidine and surface protectant after cationic surfactant treatment released the active drug in relatively lower concentration even after 1 day of desorption and then decreased slowly to 7.9μg/mℓ after 10 days of desorption. Chlorhexidine - treated specimen without surface protectant and cationic surfactant showed the active drug in higher concentration at 1 day of desorption and thereafter decreased rapidly to 9.8μg/mℓ after 10 days of desorption. However, the specimens treated with chlorhexidine and surface protectant without cationic surfactant showed higher, but slightly lower drug concentration

Table 3. Severity of bacterial contamination and plaque formation on titanium surface treated with anti - microbials

	Intra - oral exposure						
	30min	2h	day 1	day 3	day 7	day 14	day 21
Group C	0(0)	0(0)	1.0(1.0)	1.8(0.5)	2.8(1.0)	2.7(0.6)	3.0(0.8)
Group PTCV	0(0)	0(0)	1.0(1.0)	1.0(1.0)	1.8(0.5)	2.5(0.7)	2.3(0.6)
Group PTTC	0(0)	0(0)	0.5(0.6)	1.3(1.0)	1.8(1.0)	2.0(1.4)	3.5(0.7)

N=4, Values are mean(SD).
Group C ; Passivation only
Group PTTC ; Specimens treated with TDMAC and tetracycline - HCl solution
Group PTCV ; Specimens treated with TDMAC and chlorhexidine varnish

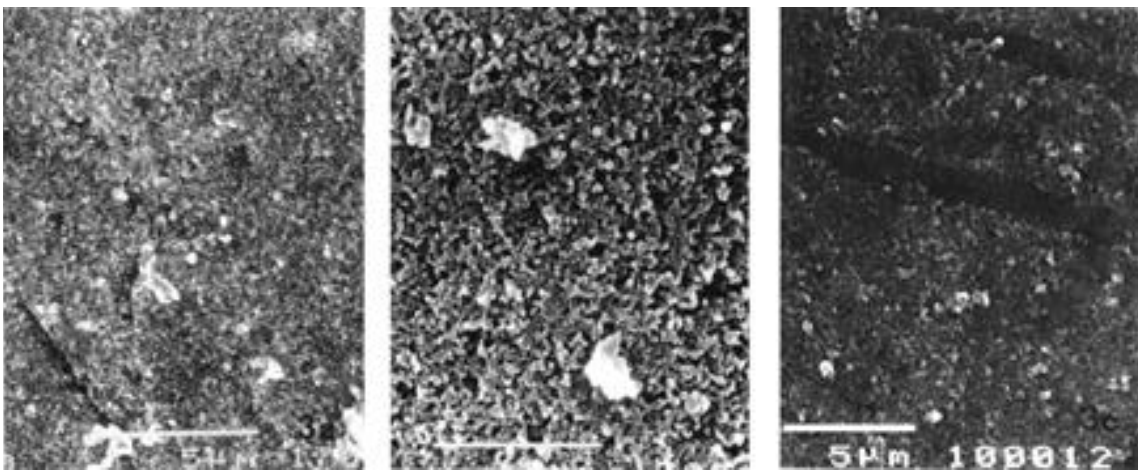

Figure 3. Specimen after 30 min and 2 hours of intra - oral exposure shows proteinaceous covering on the surface. Surface areas are free from bacterial cells.

a) Control group after 30 min of intra - oral exposure shows amorphous coating(x 5000, Bar=5 μm).
b) Chlorhexidine varnish treated group after 30 min of intra - oral exposure shows more aggregated coating compared to other groups(x 5000, Bar=5 μm).
c) Tetracycline treated group after 2 hour of intra - oral exposure shows amorphous and homogenous covering of organic materials(x 3500, Bar= 5 μm).

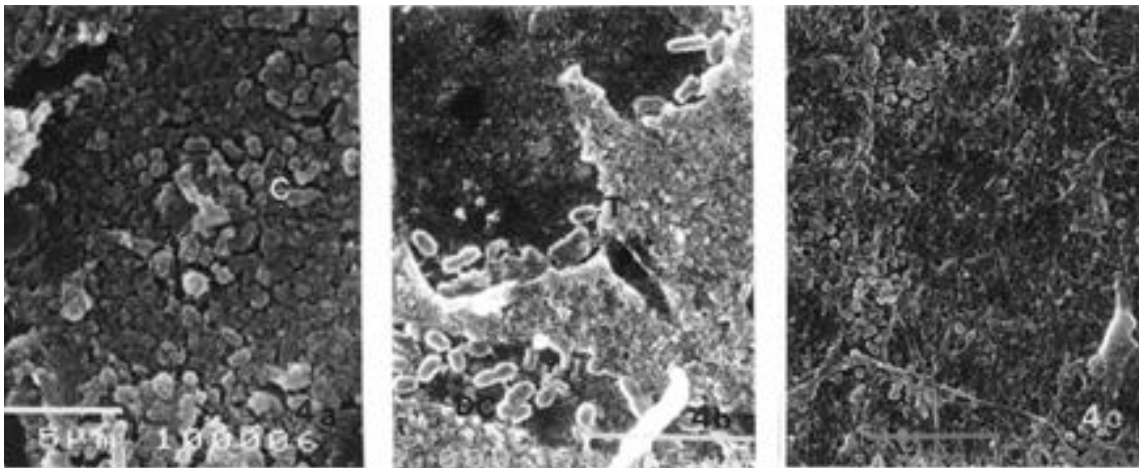

Figure 4. Specimen after 1 day of intra - oral exposure shows bacterial attachment in scattered pattern or in monolayer connected with proteinaceous covering on the surface.

a) Control group after 1 day of intra - oral exposure (x 3500, Bar= 5 µm).
The surface is colonized by cocci(C). Cocci adhere to each other or to the pellicle by their external wall. A small amount of organic material covers a few bacterial cells.

b) Other specimen in Control group after 1 day of intra - oral exposure (x 5000, Bar= 5 µm).
Early phase of colonization, dominated by cocci on the surface of an uneven pellicle. The cells are in active growth, as indicated by their frequent appearance as diplococci(DC). A glue - like material extends between single cells as threads(T) or between pellicle and bacteria as threads or a coat.

c) Tetracycline treated group. The surface is obscured by a rough pellicle (x 2000, Bar=10 µm).

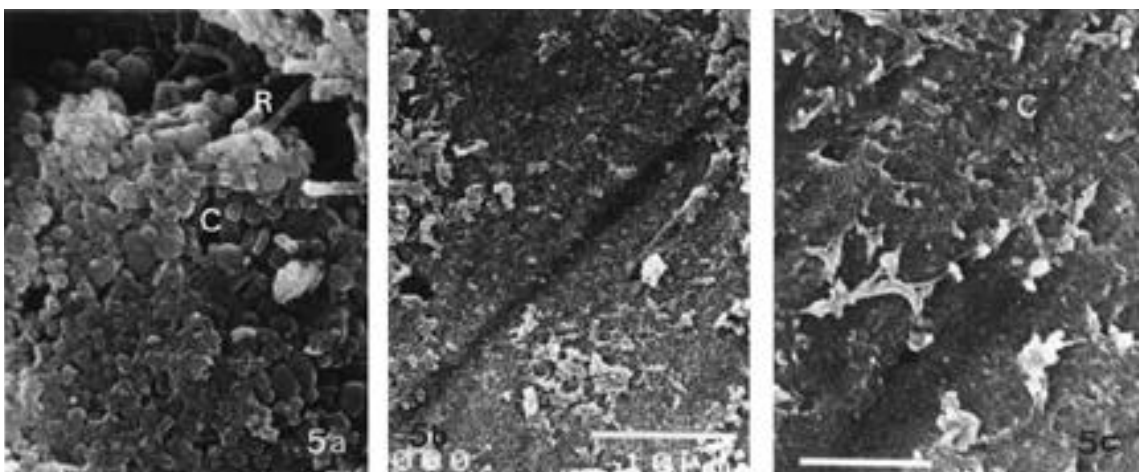

Figure 5. Specimen after 3 days of intra - oral exposure.

a) Control group shows thick multilayered bacterial attachment, predominated by cocci(C) and slight incorpo - ration of rods(R). Plaque demonstrates an increasing degree of structural complexity(x 3500).

b) Chlorhexidine varnish treated group after 3 days of intra - oral exposure shows only aggregated amorphous organic coating without bacterial attachment(x 2000, Bar= 10 µm).

c) Tetracycline treated group shows scattered bacterial attachment connected with proteinaceous covering on the surface. Bacterial colonization is dominated by cocci(C) on the surface of an uneven pellicle (x 3500,

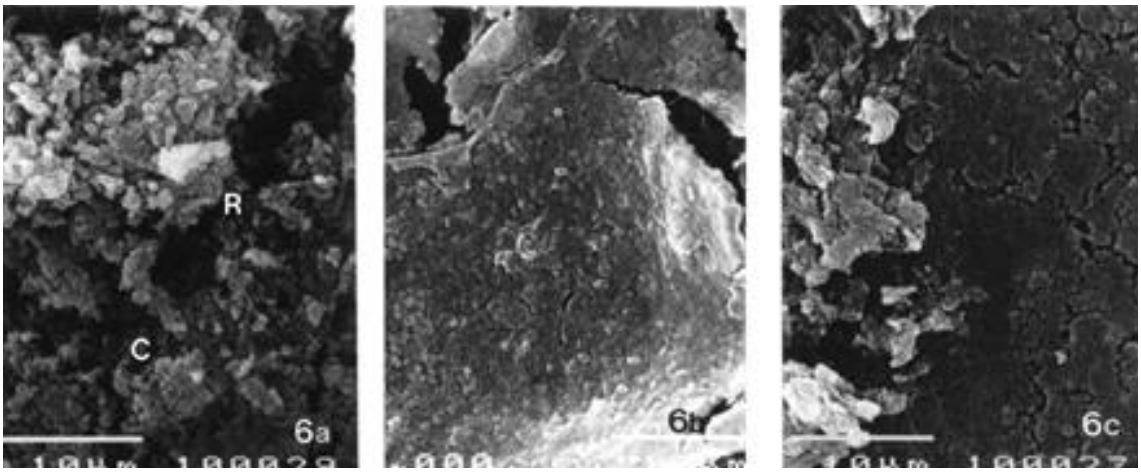

Figure 6. Specimen after 7 days of intra - oral exposure.

a) Control group shows thick and complex layer of bacterial clumps, dominated by cocci and a few rods(x 2000, Bar= 10 μm).

b) Chlorhexidine varnish treated group shows homogenous monolayer by cocci(x 2000, Bar= 10 μm). Most of the bacteria in this specimen seem to be incorporated into or covered by a masking layer. In several areas cell outlines are indistinct.

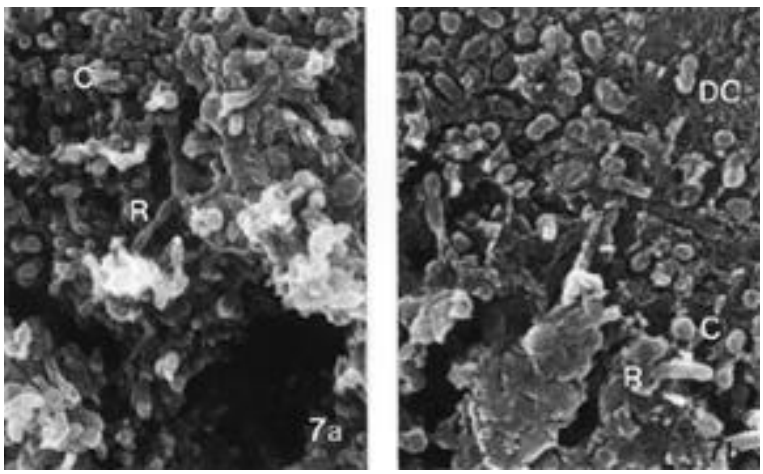

Figure 7. Specimen after 14 days of intra - oral exposure shows that the surface is fully covered by plaque. Cocci(C) are prevalent and a few small and scattered rods(R) are also present.

a) Control group(x 3500). b) Chlorhexidine varnish treated group(x 5000).

than the film treated by chlorhexidine only, after 1 day of desorption and thereafter decreased slowly to 21μg/mℓ after 10 days of desorption(Figure 1).

The anti - microbial activity of the treated films after desorption during experimental periods differed among the drugs and the treatment methods. In the tetracycline - treated films, the anti - microbial activity retained prior to desorption was equivalent

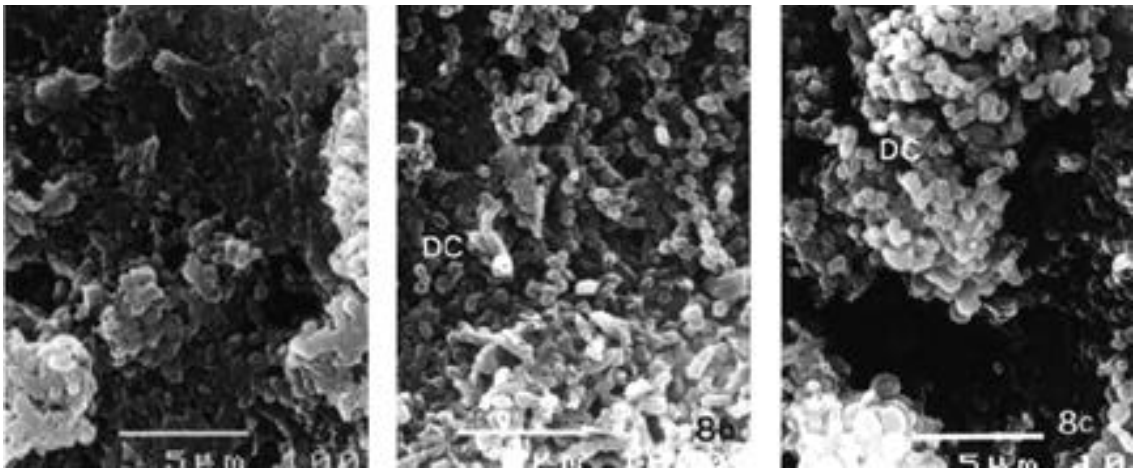

Figure 8. Specimen after 21 days of intra - oral exposure shows thick and compact layer of bacterial clumps that cocci are prevalent and a few small and scattered rods are also present.

a) Control group(x 3500, Bar= 5㎛). Most of the bacteria in this specimen seem to be incorporated into or covered by a masking layer.In several areas cell outlines are indistinct.

b) Chlorhexidine varnish treated group(x 3500, Bar= 5㎛). The cells are in active growth, as indicated by their frequent appearance as diplococci(DC).

c) Tetracycline treated group shows bacterial aggregates resembling clusters of grapes(x 3500, Bar=

to 5400~ 9500㎍ /㎖ . The activity decreased rapidly to undetectable level after 2 weeks of desorption from the specimen with tetracycline only and decreased relatively slowly to 11.8㎍/㎖ after 3 weeks and 1㎍/㎖ after 4 weeks in the specimen treated with tetracycline and cationic surfactant. This activity was in the range higher than the level known to inhibit periopathogen.

The retained anti - microbial activity of chlorhexidine treated films differed according to the treatment methods. Regardless of pre - treatment of the cationic surfactant, the films with chlorhexdine and surface protectant showed 58~68㎍/㎖ prior to desorption and the films treated with chlorhexidine only, 76~198㎍/㎖ of anti - microbial activity. Compared to the baseline prior to desorption, the activity of films exposed in desorption media showed higher during the whole experiment periods(from 1 day to 28 days) and was lower in films treated with surface protectant than the films without it. In the films treated with chlorhexidine and surface protectant, the anti - microbial activity increased at the first day of desorption and gradually decreased to the level(51~439㎍/㎖), still much higher than baseline in the film with prior cationic surfactant, after 4 weeks of desorption. However, the films treated with chlorhexidine only showed the highest level during 7 days of desorption and then gradually decreased to the level(309~487㎍/㎖), still twice higher after 4 weeks of desorption, compared to the baseline level.

In the case with surface protectant over chlorhexidine, the anti - microbial activity of the films during the desorption periods was

different according to previous treatment of cationic surfactant. The anti-microbial activity in the film, without cationic surfactant, decreased rapidly to 51μg/mℓ after 4 weeks of desorption, whereas the activity in the film with cationic surfactant was much higher than the film without it in the early period of desorption and decreased to the level(439μg/mℓ), still much higher than the baseline(Figure 2).

2. Intra-oral plaque inhibition assay

All the surface of titanium disks, after 30 min of intra-oral placement, showed no bacterial attachment except partial or generalized surface coating with amorphous or granular substances which seems to be derived from salivary proteins. Titanium surfaces of some specimens after 2 hours' intra-oral exposure following tetracycline treatment showed the proteinaceous coating similar to the 30 min's specimen, and the other surfaces were partly covered with monolayer of a few(sparsely scattered) coccal bacteria.. In the controls, an increase in bacterial count was seen between 2 and 24 h, whereas the increase with chlorhexidine was slight. The pellicle was similar in the control and chlorhexidine groups of plaque samples, but a surface coating with areas of electron-dense granular material was more often found in the chlorhexidine specimens. After 1 to 3 days' exposure, the bacterial accumulation became thicker in part, and after 7 days' exposure the overall surface was covered with bacterial layer predominated by cocci and a few rods. At 1 to 14 days of exposure, the disks which were treated with tetracycline and chlorhexidine after cationic surfactant/chloride doping agent showed relatively less(but statistically insignificant) and more slow plaque accumulation, compared to the control disks. After 21 days' exposure, all of these three groups showed similar pattern and amount of bacterial plaque on the titanium surface(Table 3, Figure 3~8).

IV. Discussion

Increased use of root-form implant in dentistry drives the clinicians to find out how to prevent and control the peri-implant tissue inflammation. It was reported that the size as well as the composition of inflammatory lesions in the periodontal and peri-implant tissues had many features in common [16~18]. Lindhe et al.[19] reported that, in ligature induced peri-implantitis, tissue destruction was more pronounced, the size of the soft tissue lesion was larger in implants than in teeth, and the lesion also extended into the bone marrow in implant sites. Nakou and his colleagues[1] reported that bacteria known as potential periodontal pathogens colonize on surface of the permucosal implants in the first week after insertion and the presence of these species seems to be dependent on the ecologic factors provided by the artificial gingival crevice of the implants in the edentulous mouth. Leonhardt and his colleagues[20] observed that the plaque formed in the pockets was similar at tooth and implant sites and was dominated by Gram(-) bacteria(*P. gingivalis* and *P. intermedius* comprise 25% of the flora). Van Steenberghe and his colleagues[3]

pointed out the importance of the plaque inhibition to prevent mucosal inflammation around dental implant. Indeed, it is very important to prevent bacterial contamination for the long term success of dental implant.

In the 2 stage implant system and the retrievable abutment system, replacing new sterile abutment or regular cleansing has been considered as good modality to control the plaque contamination. Recently, it was reported in this 2 stage implant system that the frequent removal of abutment for cleansing or abutment shift caused to break the connective tissue attachment and resulted in continuous proliferation and apical migration of junctional epithelium[21]. Ericsson and his colleagues[2] reported that an inflammatory cell infiltrate(so called abutment ICT) was consistently present at the level of borderline between the abument and the fixture part of the implant and suggested that the development of this abutment ICT is not related to the presence of plaque at the marginal border of peri-implant mucosa. The presence of abutment ICT indicates that micro-organisms probably reside within the inner part of the implant system used. Taversy and Birek[22] demonstrated *in vitro* that there was a leakage of fluid and transmission of *St. sanguis* along the fixture-abutment assembly of the 2 stage implant system. Quirynen and Van Steenberghe[23] also demonstrated that all screw harbored a significant quantity of micro-organisms and suggested that the abutment-fixture interface in the 2 stage implant system might provide niche for bacterial accumulation and result in persistent inflammatory cell infiltration around this gap area at sites which are exposed to plaque control. Therefore, to maintain the clean interfacial environment by applying slow delivery anti-microbials to the base of abutment at the abutment-fixture junction is considered as one of possible modalities to control.

Chlorhexidine and tetracycline HCl are known to have "substantivity" and adsorb to oral surfaces, including the teeth, and then are slowly released in active form. Early report[8] on the effect of chlorhexidine rinses on the morphology of early dental plaque showed that, an increase in bacterial count was seen between 4 and 24 h in the controls, whereas the increase was slow and slight in chlorhexidine group. Surface coating with areas of electron-dense granular material seems more often found in the chlorhexidine specimens. This appearance of the pellicle is possibly due to the adsorption of the chlorhexidine to the glucoproteins.

Most of recent studies focused to develop the slow release system which can release the drug in the effective concentration in the disease site for the sufficient period to inhibit the bacterial growth as the primary cause of periodontal and dental disease without adverse systemic effect. Balanyk and Sandham[12] developed Chlorhexidine varnish system and reported that the application of varnish over the tooth surface reduced the count of *St. mutans* in oral cavity[24,25]. In Korea, Chang and his colleagues[26] reported that application of this varnish system reduced the number of *St. mutans* in saliva of the orthodontic patients.

In order to inhibit the bacterial contami-

nation and plaque formation on the surface of dental implant, Holen and his colleagues[13] treated the titanium surface with TDMAC as cationic surfactant and chloride doping agent and subsequently applied tetracycline HCl. They reported that adsorption of tetracycline was improved on the TDMAC-treated surface and finally reduced the attachment and contamination of plaque bacteria on the surfaces.

Present study was done to determine the pharmaco-kinetics of the chlorhexidine varnish system and tetracycline HCl treatment on the titanium surface following pre-treatment with cationic surfactant and chloride doping agent and to evaluate whether these system could inhibit bacterial attachment and plaque formation, prior to the clinical use for prevention of plaque accumulation around dental implant and abutment system.

In the first part of study to evaluate the dynamics of drug desorption, we used artificial saliva(Arvidson and Johansson[14]) as desorption media to extract the drug from the treated film. The anti-microbial activity released into desorption media differed among the drugs and the pre-treatment methods. The inhibition diameter by anti-microbial activity of the desorbed media and the films was evaluated using *B. cereus* for tetracycline as described by Walker et al.[27]. As for chlorhexidine, Nuuja et al.[28] observed that *St. mutans* appeared more sensitive to chlorhexidine than *St. sanguis* and the diameter of the inhibition zone was 15.5mm at 1 mg/㎖(0.1%, 1.6mM of chlorhexidine diacetate) for this bacteria and 11.0mm at 0.1 mg/㎖(0.01%). Based on these data, we used *St. mutans* to evaluate the anti-microbial activity in drug desorption dynamics.

After being immersed in 50 mg/㎖ tetracycline HCl, the films has the different baseline anti-microbial activity prior to desorption and released the effective anti-microbial activity in successive desorption media for different periods of desorption, depending on the type of previous treatment. The specimens treated with tetracycline solution only without the TDMAC pre-treatment had the baseline anti-microbial activity equivalent to 5400μg/㎖ and released the drug in the highest concentration during the first day of immersion, thereafter the anti-microbial activity decreased rapidly to 2μg/㎖ in 10 days and to undetectable level after 2 weeks of desorption, whereas the specimens treated with tetracycline HCl after cationic surfactant/chloride doping agent has the baseline anti-microbial activity equivalent to 9500μg/㎖, prior to desorption and showed persistent release of the drug in the concentration above the level of 1μg/㎖ in 18 days. The activity of the film itself decreased rapidly to undectable level after 2 weeks of desorption with tetracycline only, and decreased relatively slowly to 11.8μg/㎖ after 3 weeks and 1μg/㎖ after 4 weeks with tetracycline and cationic surfactant. These concentrations are above the lowest concentration of this drug, in which the perio-pathogenic bacteria are inhibited. This result agrees with Holen and his colleague's findings[13] in that the tetracycline adsorption was improved on the TDMAC-treated surface and the drug released in

effective concentration for longer than 2 weeks and the films showed effective anti-microbial activity after 3~4 weeks of desorption.

Chlorhexidine was reported to inhibit several subgingival plaque isolates, particularly of *P. gingivalis, P. intermedius, sputigena, H. aphrophilus, A. actinomycetemcomitans, F. nucleatum* and *W. recta* with MICs ranging from 0.0006~0.01%(6~100 μg/mℓ)[29]. Stanley et al [30] also reported that the MIC of chlorhexidine for 52 strains of subgingival bacteria ranged from 8 to 500μg/mℓ, with the modal value being 62μg/mℓ. Among the strains tested, *St. mutans* and *H. aphrophilus* showed lowest MICs(<8μg/mℓ), *A. actinomycetemcomitans, P. gingivalis, P. intermedius, E. corrodens* showed intermediate MICs(31~62μg/mℓ) and *St. sanguis, A. viscosus, F. nucleatum, Capnocytophaga* species showed greater MICs(>125μg/mℓ). According to Briner et al.[31], MIC for the periodontal pathogen was 0.5~64μg/mℓ of chlorhexidine gluconate, and in Zimmermann and Preac-Mursic's report[32], the minimum inhibitory and minimum bactericidal concentrations of chlorhexidine digluconate on 10 potential oral pathogenic bacterial species were 30~120μg/mℓ. In addition, it was reported that pre-treatment of bacterial cells with chlorhexidine(>20μg/mℓ) and incorporation of chlorhexidine markedly inhibited the ability of *P. gingivalis* to adhere to epithelial cells[33].

In our experiment, the intact film treated with chorhexidine and/or surface protectant showed the baseline anti-microbial activity equivalent to 58~198μg/mℓ and than increased to more anti-microbial activity equivalent to approximately 1 mg/mℓ or more at the first day of desorption, much higher than the baseline level. The anti-microbial activity in the desorption media released from the treated film was the highest at the first day of desorption and decreased with the experiment periods. The anti-microbial activity of the treated films and the desorption media differed according to the treatment methods. The films treated with chlorhexidine only without prior cationic surfactant had the baseline anti-microbial activity equivalent to 76 μg/mℓ, increased to the highest activity(884~1004μg/mℓ) during the 1st week of desorption and decreased to 310μg/mℓ after 4 weeks of desorption, These were effective activity level, according to Stanley and his colleague's report[30] The activity in the desorption media was decreased to approximately 10μg/mℓ after 10 days of desorption. The films treated with chlorhexidine and surface protectant without cationic surfactant had the baseline anti-microbial activity equivalent to 68μg/mℓ, increased to the highest activity(880μg/mℓ) after 1 day of desorption, which were lower than the films treated with chlorhexidine only, and decreased to 52μg/mℓ after 4 weeks of desorption, which was still effective anti-microbial activity level and lower than the films treated with chlorhexidine only. However these films after 1 day of desorption released slightly lower anti-microbial activity than the films treated with chlorhexidine only and thereafter, and the activity decreased slowly to 21μg/mℓ after 10 days of desorption. It could be expected that the drug release and the activity in the

film after the experimental periods of desorption might be much higher in the oral cavity than *in vitro* release, because the anti-microbial activity under the layer of varnish could be released partly after mechanical abrasion during tooth brushing or function in the oral cavity.

The films treated with chlorhexidine only after cationic surfactant pre-treatment had the baseline anti-microbial activity equivalent to 198μg/mℓ, increased to the highest activity(1330 ~1202μg/mℓ) during the 1st week of desorption and decreased to 487μg/mℓ after 4 weeks of desorption. This film released the anti-microbial activity in the highest concentration among the 4 groups and the activity in the desorption media was decreased to approximately 21μg/mℓ after 10 days of desorption. The films treated with chlorhexidine and surface protectant over prior cationic surfactant had the baseline anti-microbial activity equivalent to 58 μg/mℓ, increased to the highest activity(1927μg/mℓ) during the 1st day of desorption and decreased to 439μg/mℓ after 4 weeks of desorption. They released the active drug in relatively low concentration(69μg/mℓ) even at the first day of desorption and then decreased slowly to 7.9μg/mℓ after 10 days of desorption.

In the case with surface protectant over chlorhexidine, the level of anti-microbial activity was different between the films with and without the prior treatment(of cationic surfactant). The anti-microbial activity in the film, without cationic surfactant, decreased rapidly to 51μg/mℓ after 4 weeks of desorption, whereas the activity in the film with cationic surfactant was much higher than the film without it in the early period of desorption and decreased to the level(439μg/mℓ), still much higher than the baseline level. In this specimen, the anti-microbial activity might be retained under the surface protectant for the relatively long periods.

This suggests that application of surface protectant over active drug leads to slow and continued drug release from treated film and higher and sustained retention of anti-microbial activity on the film. Chlorhexidine itself seems to have affinity to the Ti surface and the interaction between chlorhexidine and the Ti surface seems to be enhanced following prior treatment by cationic surfactant. While the activity from chlorhexidine-treated film was effective only for the 1st day of desorption, the anti-microbial activity of film after 4 weeks of desorption was higher than the modal value of the MIC for subgingival periodontal pathogenic bacteria[30] and higher than the concentration in which the ability of *P. gingivalis* to adhere to epithelium is affected[33], except for the chlorhexidine and surface protectant treatment without cationic surfactant.

Staboltz et al.[34] showed that the roots immersed in 50 mg/mℓ tetracycline HCl released anti-microbial activity to successive desorption media for 14 days and the roots subjected to chlorhexidine digluconate released anti-microbial activity for 24 h only, Compared to their study, our study on the titanium film demonstrated that there was higher and more slow release in effective anti-microbial activity for longer period in chlorhexidine treated film, and more

than 2μg/mℓ for 18 days in tetracycline HCl treated film with prior TDMAC conditioning. According to the results from this study, we can expect that periodontal pathogen could be affected by the chlorhexidine - treated film itself until the end of experimental period of 4 weeks and bacterial attachment and contamination on the chlorhexidine - treated titanium surface could be reduced even after 4 weeks of desorption. We need to extend the study period to evaluate how long the anti - microbial activity remains in the films after desorption.

As the clinical trials are needed to confirm the clinical significance of this *in vitro* observation, the 2nd intra - oral experiment was done to evaluate the anti - plaque activity by the anti - microbial treatment of titanium disks. All the surface of titanium disks, after 30 min of intra - oral placement, showed no bacterial attachment except partial or generalized surface coating with substances which seems to be derived from salivary proteins. Titanium surfaces of some specimens after 2 hours' intra - oral exposure following tetracycline treatment showed the proteinaceous coating similar to the 30 min's specimen, and the other surfaces were partly covered with monolayer of a few(sparsely scattered) coccal bacteria. According to Lie's study[35] on bacterial colonization of the hydroxyapatite splint on low - sucrose diet, 2 h specimen revealed incomplete pellicle covering without bacteria, bacteria were first encountered in 4 to 6 h specimen, and cocci dominated in numbers in all phases of the experiment although rods and filaments appeared in increasing amounts in 24~48 h samples. Carrassi and his colleague[36] examined the human cementum slab glued to orthodontic bracket, positioned on the upper tooth, under SEM after 2~24 h of intra - oral placement. Within 2 h, a thin pellicle covered the surface with few microorganisms detectable. At 4 h, coccoid plaques were present and the surface was completely obscured by dense networks of microorganisms after 8 h and filaments inserted perpendicular to the plaque surface were seen at 24 h. Compared to these studies, the plaque formation on the titanium surface were similar to the plaque formation on the tooth surface, but the tetracycline treatment tends to result the less plaque formation on the Ti surface. After 1 to 3 days' exposure, the bacterial accumulation became thicker in part, and after 7 days' exposure the overall surface was covered with bacterial layer predominated by cocci and a few rods. At 14 days of exposure, the disks which were treated with tetracycline after cationic surfactant/ chloride doping agent showed significantly less plaque accumulation, compared to the control disks. After 21 days' exposure, all of these three groups showed similar pattern and amount of bacterial plaque on the titanium surface. On the basis of studies with medically important bacteria, it has been established that bacteria in biofilms are less susceptible to anti - microbial agents than their planktonic counterpart[37]. According to Millward and Wilson[38], the biofilms were found to be more resistant to the bactericidal effects of chlorhexidine than the bacterial suspensions with the MICs being 25 and 50μg/mℓ, respectively, for the suspension and the 24

h biofilm and older(72 h old) biofilms tend - ed to be more resistant to chlorhexidine than younger(24 h old) ones, with the MICs being 200 and 50μg/㎖, respectively.

Generally, the bacterial score on the tita - nium surface tends to differ by the surface anti - microbial treatments in the earlier period until 2 weeks of intra - oral exposure, and thereafter there was no difference in later periods. There was some inter - indi - vidual variation rather than inter - group difference. It was considered that this was because of the different level of motivation or variation in the attitudes for the subjects to participate in this study and to stick to the instruction given.

Finding from our study agrees with some biofilm study in which bacteria in biofilms(especially older one) are less sus - ceptible to anti - microbial agents than their planktonic counterpart. This means that we should be careful to interpret the result on the antibacterial susceptibility of the bacte - rial suspension and apply this to biofilm *in vivo*. Costerton et al [39] reviewed that device - related infections can be prevented by careful cleaning and sterilization of the device, and by the avoidance of any manip - ulations that would allow the formation of even the most rudimentary biofilm prior to implantation. Once a device - related infec - tion has become established, both the MIC and the biofilm eliminating concentration(BEC) of the causative organism must be determined and thera - peutic strategy must aim at the use of the MIC to control the acute phase caused by planktonic bacteria and of the BEC to elimi - nate the biofilm nidus of infection.

Our findings suggest the usage of tetra - cycline HCl and chlorhexidine varnish after prior TDMAC conditioning on the abutment surface as a depot for sustained release of anti - microbial activity to the sub - peri - implant environment. More sophisticated clinical trials are needed to confirm the clinical significance of these *in vitro* and *in vivo* observations.

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- Abstract -

항균제 처리한 titanium 표면의 치태형성 억제효과

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매식된 인공치아의 성공을 위해서는 적절한 교합과 수동적 적합성을 갖는 보철물의 제작과 구강내 노출 직후부터의 세균성 치태조절이 요구된다. 본 연구는 전처리(passivation과 tri - d o d e c y l - m e t h y l - a m m o n i u m chloride(TDMAC) 처리)가 다른 타이타늄 표면에 chlorhexidine varnish와 테트라사이클린을 도포시 약제의 방출역학을 알아보고 구강내 치태형성의 억제정도를 평가하기 위하여 시행되었다. 이를 위해 방출용액으로 인산완충액 성분의 인조타액을 1일~1개월간 매일 교환하여

약제농도를 측정하고 타이타늄 박막에 잔류한 약제 활성을 측정하였으며 항균제 도포한 타이타늄 원판을 부착한 장치를 구강내 위치시킨 1일~3주 후 원판을 제거하여 주사전자현미경으로 세균 부착상을 관찰하였다.

테트라사이클린은 TDMAC 처리된 표면에서 10~18일까지 유효농도로 방출되었고 표면의 유효 항균 활성은 3~4주간 유지되었으며, chlorhexidine varnish 도포 시에는 TDMAC 전처리시 초기에 3~7일 간 증가한 유효 항균 활성을 방출하여 매식지대치 등에 이러한 항균제도포 시 매식치 주위환경에 항균활성 공급원으로 작용할 수 있음을 보였다. 주사현미경적 관찰시 모든 타이타늄 표면에서 구강내 위치 30분 후에는 세균이 부착되어 있지 않고 타액 단백질 성분에서 유래한 것으로 보이는 피막물질이 표면을 부분 또는 전면에 걸쳐 덮고 있었다. 구강내 노출 2시간 후 항균제 미도포 표본들에는 약간의 구균이 단층으로, 1~3일 후에는 부분적으로 두꺼운 세균층을 형성하였고 7일 후에는 표면전체에 걸쳐 세균층이 덮여있었으며 주로 구균과 약간의 간균이 주종을 이루었다. 항균제 도포시 구강내 노출 1주일 이전까지는 미도포군에 비해 치태형성이 지연되는 경향을 보였지만 2주 이후에는 세균 수나 치태형성 양상이 유사하였다.

이 연구로부터 항균제 도포시 1주일 이전의 초기 치태형성을 감소시킬 수 있음을 알 수 있었으며 이러한 연구결과는 타이타늄 임프란트 지대치 표면에 항균제의 도포가 임상적으로 유용할 수 있음을 시사하였다.