

The Effect of Chlorhexidine on the formation of bone nodules by Periodontal ligament Cells in Vitro

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I. Introduction

Chlorhexidine(CHX) is a cationic molecule belonging to the polybiguanide group of compounds. The bactericidal effect of the drug is the result of the cationic molecule altering the osmotic equilibrium of the microbes¹⁾. Generally, a 0.12% CHX mouth rinse is used to prevent dental plaque formation on the teeth and other plastic films²⁾. Its use has been constantly advocated after GTR surgery to prevent or at least to reduce membrane exposure and the incidence of wound infections^{1,3,4)}. In order to prevent membrane contamination, systemic antibiotics and local antimicrobial therapy with (0.12% or 0.2%) CHX solutions have been suggested⁵⁾. Allyn et al.⁶⁾ demonstrated greater bone regeneration in the bifurcation defects in

dogs when topical CHX was used postoperatively. In addition, many studies have shown that periodontal ligament cells produce mineralized nodules in vitro⁷⁻⁹⁾.

However, several studies have demonstrated the toxic effects of CHX on human cells and granulation tissue¹⁰⁾. A review of the literature showed CHX to be harmful to a variety of mammalian cells, including sperm, polymorphonuclear leukocytes, macrophages, skin epithelial cells, erythrocytes, gingival fibroblasts and PDL cells¹¹⁻¹⁸⁾. In addition, some studies have reported that CHX application directly to surgical wounds in the oral cavity can delay and alter wound healing¹⁹⁻²⁰⁾.

Although there are no reports showing that immediate CHX mouth rinsing after GTR can approach PDL cells, it is difficult to state that

* This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (03-PJ1-PG1-CH08-0001).

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they cannot. Moreover, exposure of the GTR membrane during the healing period is common occurrence²¹⁻²²⁾, and when the membrane is exposed, a 0.12% CHX gel and forceful irrigation with CHX using irrigating syringes is the recommended method for clinics²³⁾. The use of CHX in membrane exposure might increase the possibility of contact with the PDL cells.

The concentration range of the antiseptic that causes minimal tissue damage and is still an effective antimicrobial agent has not been established. In addition, the molecular mechanisms underlying the cytotoxicity of CHX and the impairment of the function of human PDL fibroblasts are not clearly understood.

The aim of this study was to determine the optimal concentration to obtain dual effects: not harmful to human PDL cells and have a bactericidal effect on periodontal pathogens.

II. Material and Methods

1) PDL cell culture

Human PDL cells were cultured by using an explant technique that is described elsewhere^{7,18)}. The human PDL cells were cultured from the middle third of the roots of premolars extracted for orthodontic reasons. The fragments were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Experiments with PDL cells were performed by using the cells between the third and four passage.

2) Cell viability (MTT) assay

The cell viability was determined by examin-

ing the of the cells to metabolically reduce the tetrazolium salt, 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT), to a purple formazan dye, using a kit purchased from Boehringer Mannheim Corp. (Indianapolis, IN, USA). Individual wells of the 96-well microtiter tissue culture plates were seeded with 3x10⁴ cells in 0.2 ml of the growth medium and incubated overnight at 37°C. The growth medium was then removed and replaced with DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics, with various CHX concentrations (0(control), 0.12, 0.012, 0.0012, 0.00012%). The CHX(Sigma, 2.0%) was diluted with the media(DMEM +10%FBS+ 1x antibiotics). After 30, 60, 120 seconds of exposure, MTT was added to a final concentration of 0.5 mg/ml and the cells incubated for 4h at 37°C. Using the solubilization solution provided in the kit, the purple formazan crystals, which were produced in the kit, and the purple formazan crystals, which were produced as a result of the reduction of MTT by the metabolically active cells, were dissolved overnight exposure at 37°C. The absorbance was read at 490nm using a microtiter plate spectrophotometer.

3) Mineralized nodule formation

The PDL cells were seeded at an initial density of 5x10⁴ cells in 12-well microtiter tissue culture plates using DMEM with 10% FBS. Before reaching confluence, the growth medium was then removed and replaced with DMEM with various CHX concentration (0 (control medium), 0.12, 0.012, 0.0012, 0.00012%). After 30, 60 and 120 seconds, the test medium was removed, and the PDL cells were washed 2x with the HBSS solution. The PDL cells were cultured

for 21 days in DMEM containing 10% FBS, mineralization supplements, 50 μ g/ml ascorbic acid, 10mM β -glycerophosphate and 100nM dexamethasone. The medium for all groups was changed every 2 days. In order to detect the formation of mineral-like nodules, the cells were washed three times with saline and fixed with dehydrated ethanol for 20 min. The cell layers were then stained with 1% alizarin red S in 0.1% NA4OH for 5 min. The alizarin red-positive nodules on the criss crossing cell layers were defined as mineralized nodules, and the numbers of nodules per well was counted using an optical microscope.

4) MIC

Seven consecutive distinct clinical isolates of *Fusobacterium nucleatum* 25586, *Porphyromonas nigrescens* 33563, *Porphyromonas gingivalis* w50, *Porphyromonas endodontalis* 35406 and *Prevotella intermedia* 25611 from the Biochemistry

Laboratory were investigated. The bacteria were identified according to standard microbiological procedures.

The MIC of CHX was determined using the method reported by Murray. The MIC was interpreted according to the National Committee for Clinical Laboratory Standards. Briefly, a concentration of approximately 5x10⁵ of the bacterial cultures was inoculated into 10–1000 serial dilutions (0.12–0.00012%) of CHX in a Mueller–Hinton broth for MIC detection.

5) Statistical analyses

The statistical analyses were performed using the Kruskal–Wallis Test. All experiments were performed at least three times. The graphical data is presented as the arithmetic mean percentages of the control \pm standard deviation. The differences between the control and experimental values were analyzed and a P-value ≤ 0.05 was considered significant.

Table 1. Cell cytotoxicity according to the concentration of CHX and time course

time	Number of cell (mean \pm SD)				
	control	0.12%	0.012%	0.0012%	0.00012%
30 sec	0.143 \pm 0.014	0.028 \pm 0.015*	0.144 \pm 0.005	0.222 \pm 0.047	0.185 \pm 0.034
60 sec	0.156 \pm 0.023	0.028 \pm 0.007*	0.139 \pm 0.040	0.181 \pm 0.022	0.157 \pm 0.010
120 sec	0.164 \pm 0.054	0.010 \pm 0.004*	0.184 \pm 0.012	0.207 \pm 0.060	0.160 \pm 0.019

* significant difference(p<0.05) between control and test group by Kruskal–Wallis Test, SD: standard deviation

Table 2. The formation of bone nodule according to the concentration of CHX and time course

time	Number of bone nodule (mean \pm SD)				
	control	0.12%	0.012%	0.0012%	0.00012%
30 sec	10 \pm 1	0*	2 \pm 0*	5.667 \pm 0.577*	6.667 \pm 0.577*
60 sec	9.333 \pm 0.577	0*	1 \pm 0*	3.333 \pm 0.577*	3.333 \pm 0.577*
120 sec	11.667 \pm 2.081	0*	0.667 \pm 0.577*	2.333 \pm 0.577*	3.333 \pm 0.577*

* significant difference(p<0.05) between control and test group by Kruskal–Wallis Test, SD: standard deviation

III. Results

PDL cell viability is shown in table 1. The cell viability was affected by exposure to only 0.12% CHX exposure. At concentrations \leq 0.012% CHX, the cell viability was not significant different than the control. Time was found to be an independent factor for cell viability.

Numerous mineralized nodules were identified as darkly stained patches in the control group, whereas an extremely small number of mineralized nodules were observed in the CHX group (Table 2).

The PDL cells cultured in the absence of CHX differentiated in four stages; confluent, multilayer, nodule and mineralization. Therefore, many mineralized nodules were produced. (Figure 1)

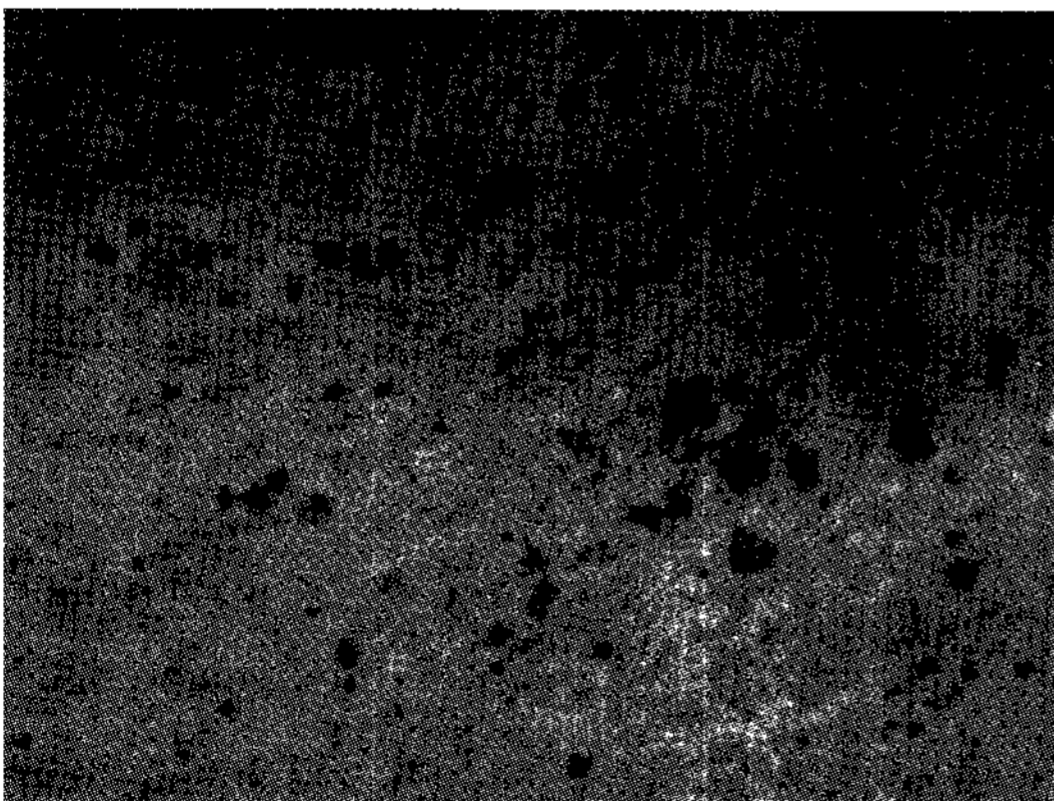


Figure 1. Numerous mineralized nodules were identified as darkly stained patches in the control group (PDL cells cultured in the absence of CHX). (Alizarin red stain, magnification $\times 100$).

The cells treated with 0.12% did not form a confluent monolayer and failed to form mineralized nodules. (Figure 2)

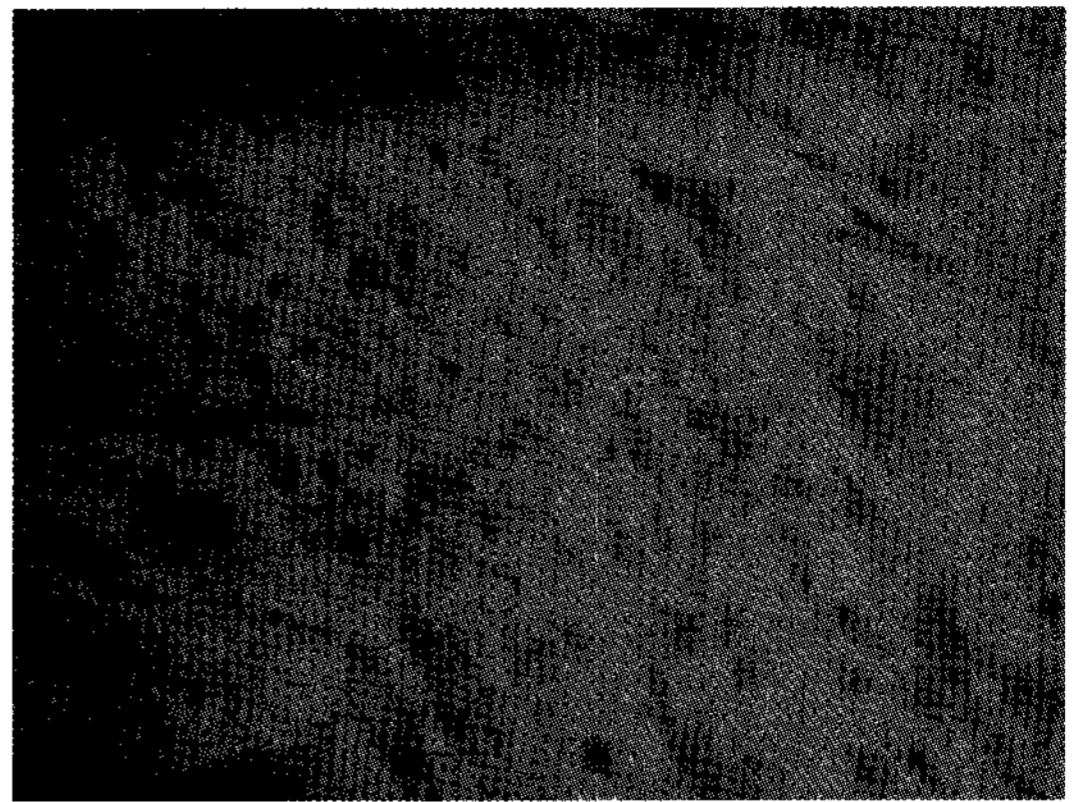


Figure 2. Scattered PDL fibroblasts did not form the mineralized nodules in the group treated with 0.12%–30 seconds CHX. (Alizarin red stain, magnification $\times 100$).

However, a small number of mineralized nodules were present in the 0.012–0.00012% CHX groups. PDL cells in certain areas also proliferated and formed multilayers of fibroblastic cells but they remained the same thereafter without further differentiation into the nodules and mineralization stages. (Figure 3)

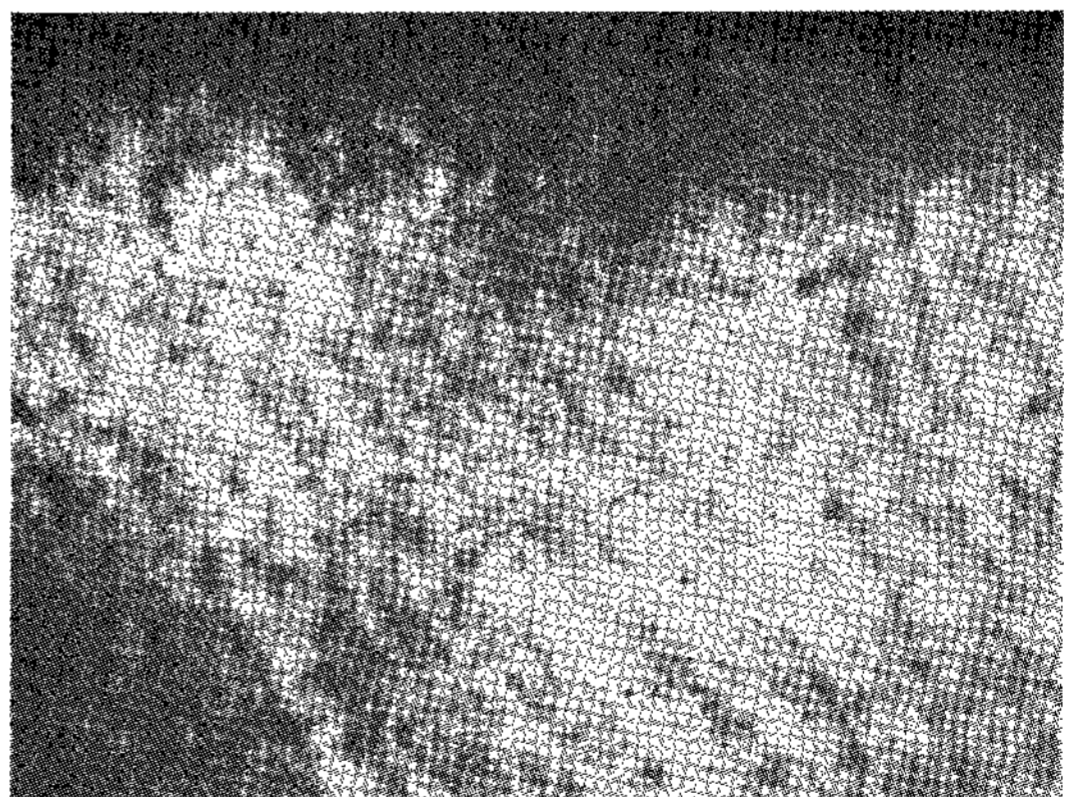


Figure 3. PDL cells in certain areas proliferated and formed multilayers of fibroblastic cells but remained the same thereafter without further cell differentiation into the mineralized nodules in the group treated with 0.012%–30 seconds CHX. (Alizarin red stain, magnification $\times 100$).

The PDL cells in some parts proliferated and differentiated into mineralized nodules. (Figure 4,5)

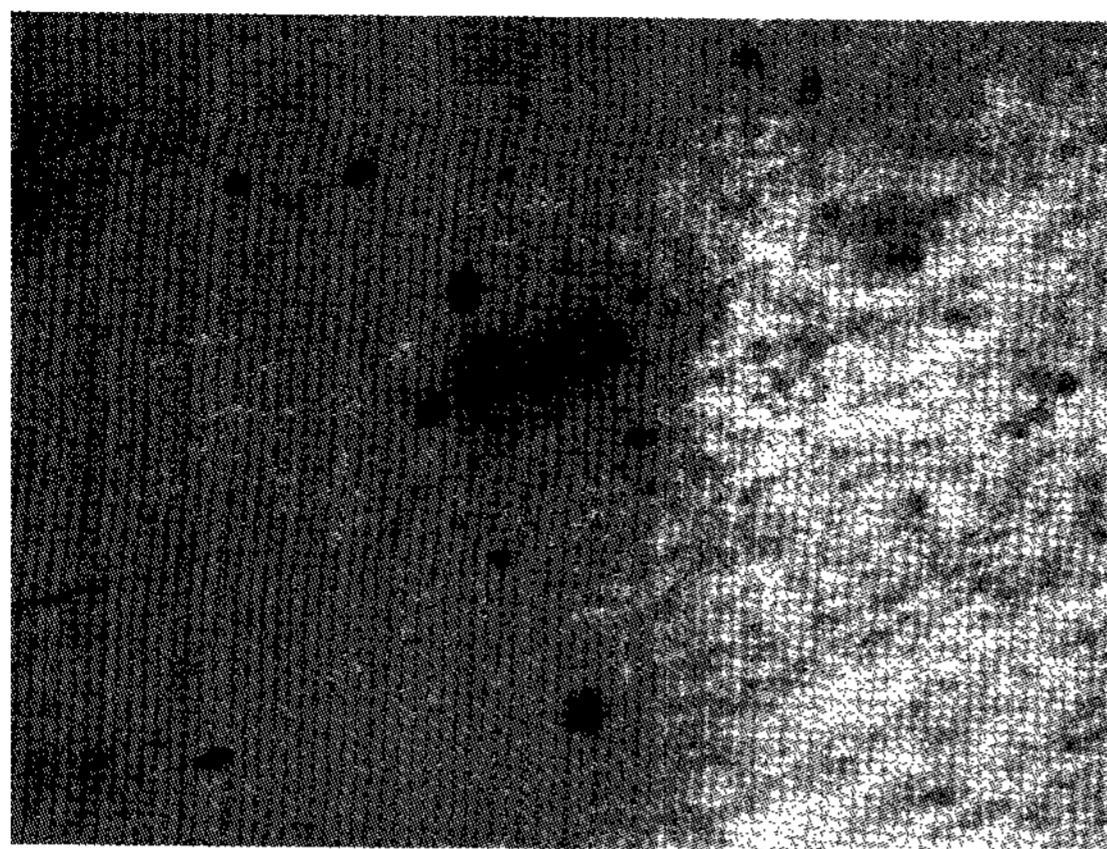


Figure 4. PDL cells in certain areas proliferated and differentiated into the mineralized nodules in the group treated with 0.0012%–30 seconds CHX. (Alizarin red stain, magnification $\times 100$).

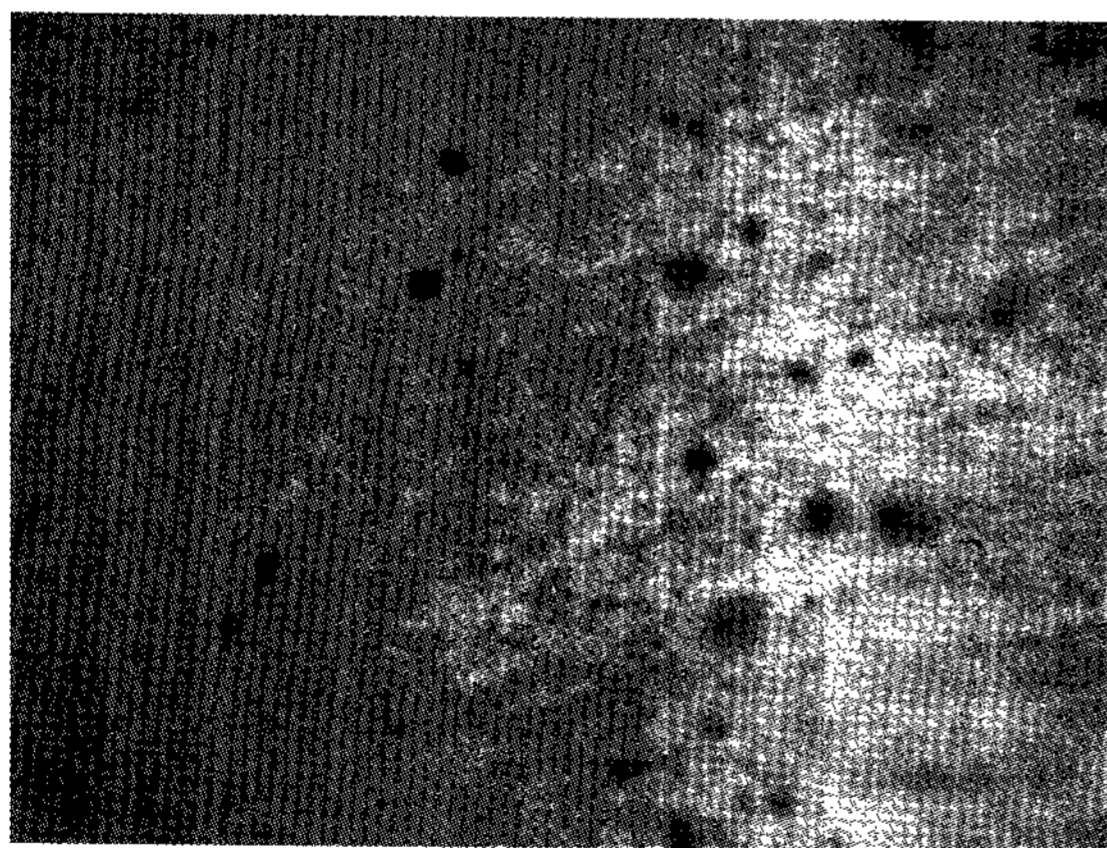


Figure 5. PDL cells in more larger areas than 0.0012% group proliferated and differentiated into the mineralized nodules in the group treated with 0.00012%–30 seconds CHX. (Alizarin red stain, magnification $\times 100$).

The MIC of CHX for the *Fusobacterium nucleatum* 25586, *Porphyromonas nigrescens* 33563, *Porphyromonas gingivalis* w50, *Porphyromonas endodontalis* 35406, *Prevotella intermedia* 25611 was found to be 0.0012%

IV. Discussion

A CHX mouth rinse is a widely used adjunct to in periodontal therapy owing to its bactericidal effect⁴⁾. There are numerous reports on its safety as an oral rinse, but its reported effects on wound healing have been contradictory. Several reports on gingival wound healing concluded that the drug did not interfere with healing^{24–26)}. However, many studies reported a significant delay in palate mucosa–osseous wound healing after applying 0.5% CHX application²⁰⁾. If CHX can come into contact with the periodontal ligament cells during the initial stages of healing, it is possible that the drug may adversely affect the regeneration of the attachment apparatus. This of course would be particularly true for GTR. To date, there is a dearth of information defining the lethal CHX dose, and perhaps more importantly, the effects of non-lethal doses on the secretory phenotype (bone nodule formation) of human PDL cells has not been investigated.

This study found there to be some CHX protective mechanism in the organism, because CHX concentrations, which are highly cytotoxic in vitro generally, do not harm cells in vivo. The epithelial barrier function can protect the underlying cells²⁷⁾. It has been shown that the serum protects cells from the cytotoxic effects of CHX¹²⁾. It has also been suggested that there is a threshold value of tissue injury in the wound that must be exceeded before the repair process is impaired²⁸⁾. Studies have found that in gingivectomies, the surface of the wound becomes covered with a fibrin clot, in which the PMNs become incorporated form–

ing a "polyband" layer. Epithelial cells migrate to cover the wound under this layer. The "polyband" layer may offer a protective surface that prevents CHX from reaching the connective tissues²⁵⁾. In order to reflect these CHX protective mechanisms in organisms, the cells were exposed to low concentrations (0.12–0.00012%) and for a short time (30–120 seconds). The results showed that the MIC of CHX on periodontal pathogens is 0.0012%. That is a 100 dilution of the clinically used concentration (0.12%).

In the cell viability test, only 0.12% CHX affected cell proliferation. In many other studies, the cytotoxic concentration of CHX on fibroblasts was lower than but the exposure time was more than 360 times that in this study. Another reason for our result being higher than that reported elsewhere may be the difference in the composition of the media used. The media used as diluent of CHX (Sigma, 2.0%) in this study was DMEM +10%FBS+ 1x antibiotics. Hidalgo et al.²⁹⁾ reported that 10% FBS added to the culture media appeared to have an attenuating effect against CHX-induced cytotoxicity, which resulted in a higher cell survival rate, a higher ATP intracellular level and a higher rate of DNA synthesis.

Recently CHX was found to impair the cell function of the target cells at concentrations that had no effect on cellular viability. Many reports strongly suggest that CHX can inhibit the formation of mineralized bone nodules via different mechanisms, possibly inhibiting protein synthesis, in particular collagen, DNA synthesis and the mitochondrial activity. In this study, all CHX groups showed a significant reduction in bone nodule formation by PDL

cells. Perhaps the more interesting observation was the significant reduction at CHX concentrations that had no effect on cellular proliferation. The concentration of affecting the formation of bone nodules of the PDL cells (0.00012%) was lower than the cytotoxic concentration (0.12%), and the concentration for minimal inhibition on periodontal pathogens was 0.0012%. Therefore, if CHX comes into contact with the periodontal ligament cells during the initial stages of healing after a GTR, the drug might adversely affect the regeneration of the attachment apparatus.

V. Conclusions

In conclusion, this study demonstrated in vitro that CHX adversely affects the formation of bone nodules by human PDL fibroblasts. Therefore, it may be prudent to avoid contact with CHX until wound healing is well advanced. In order for CHX to be used as an antimicrobial agent in wound healing after periodontal surgery further studies will be needed to determine when the PDL cells are free from CHX.

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사람치주인대섬유모세포에 의한 골결절 형성시 Chlorhexidine의 효과

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사람치주인대섬유모세포(human periodontal ligament fibroblast, PDLF)의 기능 손상과 클로르헥시딘 (Chlorhexidine, CHX)의 세포독성에 관한 분자적인 기전은 최근까지도 불명확하다. 이 연구의 목적은 PDLF에 의한 골결절 형성에 있어서 CHX의 효과를 평가하고, 치주수술후에 치주병원균의 최소억제농도(minimal inhibitory concentration, MIC)를 평가하고자 하였다. CHX의 세포독성을 평가하기 위하여 MTT assay법을 실시하였다. CHX은 0.12 %에서 0.00012%까지, 즉 10-1000배로 희석시킨 후 30, 60, 120초 동안 PDLF에 적용되었고, 석회화된 결절은 alizarin red 용액에 염색되었다. 치주병원균에 대한 CHX의 MIC가 평가되었다. 이 연구 결과, 세포생존을 검사에서는, 단지 0.12 % CHX 에 노출되었던 세포들만 세포 증식 소견을 다소 나타내었다. 모든 CHX 농도(0.12%-0.00012%)에서 PDLF에 의한 골결절 형성은 의미있는 감소를 나타내었다. 또한 치주병원균에 대한 CHX의 MIC는 0.0012 %로 나타났다. PDLF의 골결절 형성에 영향을 주는 농도(0.00012%)는 세포독성을 나타내는 농도(0.12%)보다 더 낮은 농도를 보였고, 치주병원균의 최소억제에 필요한 농도는 0.0012%로 나타났다. 이러한 결과들은 통상적으로 상용되는CHX이 PDLF에 의한 골결절 형성에 있어서 영향을 미칠 수 있음을 시사하였다.