Effects of Low-Level Laser Irradiation on the Rat Osteoblast Function

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Ⅰ. Introduction

Low level laser therapy (LLLT) has been used for more than a decade in clinical practice with successful results in the fields of dentistry and medicine. The therapeutic effects were generally attributed to enhanced cell proliferation[1].

Kim et al[2]. reported that LLL irradiation had a favourable influence on gingival inflammation with the change of rate in the composition of oral flora. Kim et al[3]. reported that infrared gallium-arsenide (GaAs) laser irradiation at LLLT levels stimulated the growth of *Streptococcus mutans* but the growth rate of *Streptococcus mutans* is not always in accordance with the frequency or fluence of LLLT laser. Kim et al[4]. suggested, in their study for Candida albicans, that specific laser pulses are recommanded to have the biostimulation effects on the specific tissue or cells, although the biostimulation effect is dose dependant. And it was suggested that an appropriate irradiation interval should be obtained in order to achieve acceleration fo cell growth[5,6].

It has been proposed that LLLT stimulate the protein and DNA synthesis to accelerate the proliferation of the gingival fibroblast.[7,8] Rigau et al[9]. reported that no increase in the number of fibroblasts following low reacuve-level laser therapy levels of radiation, but did reveal significant changes in metabolic rates compared with the unirradiated controls suggesting an efficient modality for enhancement or modulation of cell activity. Bolton et al[10]. reported that succinic dehydrogenase levels

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rise with fibroblast proliferation at the lower energy density (2J/cm^2). Steinlechner et al[11]. suggested that LLLT of various wavelengths has biostimulatory effect on keratinocytes. Kim et al[3]. suggested that LLLT has biostimulative effects on all kinds of cells.

The purpose of this study is preliminary to study the effect of LLLT (904 nm of GaAs semiconductor laser) on the osteoblastic function investigating the changes of alkaline phosphatase activity, bone nodules and cell growth.

Ⅱ. Materials and Methods

Isolation and culture of rat calvarial osteoblast (ROB) and ROS/17/2.8

Osteoblastic-enriched cell populations were isolated from 19 day-old fetal Sprague-Dawley rat calvaria according to the methods described by Wong and Cohn[12]. Briefly, rat calvaria were dissected aseptically with enzyme solution containing 0.1% collagenase, 0.05% trypsin and 0.5 mM EDTA. At 10, 10, 10 20 and 20 minute interval (this procedure yielded five populations of cells designated I, II, III, IV and V), released cells of populations IV and V, which were characterized as osteoblastic cells, were collected and washed 2 times with Hank's balanced salt solution (HBSS). The cells were plated in a 60 mm culture dish in minimum essential medium supplemented with 10% fetal bovine serum (MEM/FBS) and incubated for 7 days at 37℃ in 95% humidified air containing 5% CO_2. After 7 days, the cells were plated in culture dishes or culture plates according to its purposes.

A rat osteoblastic cell line ROS17/2.8 cells were kindly provided by Dr. Peter Hauschka (Children's Hospital, Boston, MA, USA). The cells were cultured in medium F-12 supplemented with 5% fetal bovine serum (F-12/FBS) at 37℃ in a humidified atmosphere of 5% CO_2 in air. The cells were passaged over every 7 days in the 60 mm culture dish.

Determination of proliferation and viability of ROB

In order to determine the effect of LLL irradiation on the cell proliferation and cell viability, ROBs were plated at a density of 2-3 X 10^{-3} cells/well in the 96-well plate. At 20-30 % of confluency, laser was irradiated to the cells for 1 min every 12 hours for 11 days. At the day of first, 4th, 7th and 11 day after LLL irradiation, the cells were harvested by trpysinization, and the viable and dead cells were counted in a hemocytometer by trypan blue exclusion test.

Measurement of alkaline phosphatase (ALP) activity

To determine the effect of LLLI on the ALP activities of osteoblastic cells, ROB and ROS17 /2.8 cells were plated into 96-well plate at a density of 2-3 X10^{-3} cells/well in MEM/FBS and F-12/FBS subsequently and cultivated until confluency was obtained. To determine the ALP activities, the cells were irradiated with laser for 1 min every 12 hours for up to 9 days and the ALP activities were assayed simultaneously from 1 day to 9 days during LLLI. After removal of the culture media from the wells, the cells were washed with DPBS twice and treated with 0.1% Triton X-100/ saline for 30 min. To assay the ALP activity, resultant cell lysate was incubated in 0.1 M glycine-NaOH buffer (pH 10.4) with 100 mM p-nitrophenyl phosphate as a substrate at 37℃

for 10 min. The optical density of p-nitrophenol, a reaction product, was read at 405 nm using ELISA reader (SLT Labinstruments, 400 SFC) spectrophotometically.

Measurement of calcified nodule formation

To determine the effects of LLLI on the calcified nodule formation through the long-term culture of osteoblastic cells, ROB and ROS17/2.8 cells were plated into 24-well plate at a density of 1-2 X10^4 cells/well in MEM/FBS and F-12/FBS subsequently and cultivated until reached confluency. After confluency, the cells were fed with media supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate and LLLI was performed during the culture. Media were changed every second or third day and cultures were maintained up to 21 days. To observe the produced calcified nodule, the cell layer was fixed with neutral buffered formalin and stained in situ by the von Kossa technique for mineral deposits. Number of mineralized nodules were counted at 40X magnification using a light microscope.

Laser irradiations

Twenty four samples for the determination of growth were randomly divided into 2 groups: control and irradiation groups. Fifty samples were used to investigate the ALP activity for ROB and ROS cells respectively. Samples to study the formation of bone nodules for ROB and ROS cells were 15 and 24.

The author used a laser beam detector to ascertain the optimum distance from the laser probe to the base of dish, because the laser beam was needed not to irradiate outside of dish base. Using a clamp laser probe was precisely positioned vertically about 2 cm above the middle of media in a dish base of 24-well plate (1.88cm^2) and 1.7 cm above a dish of 96-well plate (0,32 cm^2). Used pulse was pulse 13 (P13, 6000 Hz, 14 mW of average output power) and LLLI were performed every 12 hours for 9 days to study the proliferation and the ALP activity and for 3 days a week for 21 days. Therefore, the energy density and the total energy for studying the proliferation of ROB were 1312.5 mJ/cm^2 and 23.625 J/cm^2. The energy density and the total energy density for the formation of bone nodule were 446.8 mJ/cm^2 and 8.04 J/cm^2.

Statistical analysis

All measurements in each group were averaged. Statistical comparisons were then made. To determine a significance of effects on the growth of ROB after LLLI, two factor ANOVA test was used. To compare the effects of LLLI according to the irradiation duration, Fisher's Protected Least Significant Difference (PLSD) test was used and to detect the difference of bone nodule formation between ROB and ROS group after LLLI, unpaired t-test was used.

III. Results

Effect of LLLI on the proliferation and viability of ROB

To study the effects of LLLI on the proliferation and viability of ROB cells, cells were treated with LLLI for 1 min every 12 hours for 11 days. Table 1 and 2 show the means and standard deviations of all groups and the results of ANOVA and mutiple comparison tests. LLLI increased slightly the cell prolife-

Table 1. Effect of LLLI on the proliferation of ROB (X10^{4} cells/ml)

Group	Day1		Day4		Day7		Day11		p value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Control	8.04	0.48	10.83	0.67	16.55	1.57	17.73	0.89	-
Experimental	9.5	1.21	12.63	1.22	17.84	1.15	19.48	0.76	
p value	-		-		-		-		

Table 2. Effect of LLLI on the viability of ROB (ratio of dead cell(%))

Group	Day1		Day4		Day7		Day11		p value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Control	22.3	3.21	6	3.46	4.55	2.4	3.63	1.95	-
Experimental	20	8.89	4.67	3.21	2.86	1.28	0.84	0.73	
p value	-		-		-		-		

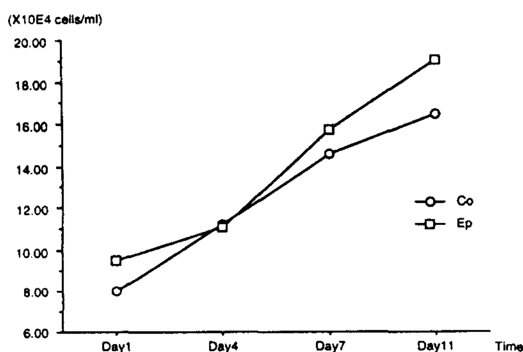

Figure 1. Linear gram showing the growth change of ROB with time

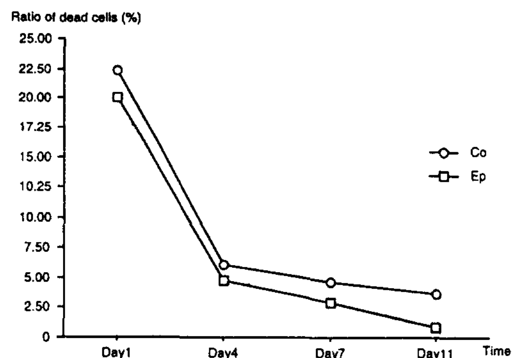

Figure 2. Linear gram showing the growth change of dead cells

ration with time(Figure 1). There was, however, no significant difference statistically among the groups. In addition there was no significance statistically, but LLLI decreased the dead cells with time (maximally 3.63 dead cells in control group vs 0.84 in LLLI group)(Figure 2).

Effects of LLLI on the ALP activities of ROB and ROS17/2.8

To determine whether LLLI affects on the ALP activity of ROB and ROS17/2.8 cells, the cells were treated with LLLI in 0.4% FBS

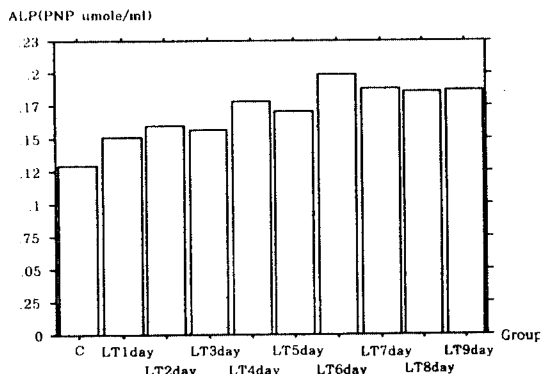

Figure 3. Histogram showing the differences of amounts of ALP in all groups of ROB according to the irradiation duration

Table 3. Means and standard deviations of ALP in all groups of ROB measured according to the irradiation duration (PNP umoie/ml)

Group	Count	Mean	Std. Dev.
Co	6	.129	.012
LT1day	6	.151	.013
LT2day	6	.160	.010
LT3day	6	.157	.016
LT4day	6	.178	.011
LT5day	6	.170	.020
LT6day	6	.199	.029
LT7day	6	.188	.017
LT8day	6	.186	.015
LT9day	6	.186	.013
p value		<.0001	

Table 4. Results of multiple comparison test for all groups of ROB according to the irradiation duration

	Co .129	1d .151	2d .160	3d .157	4d .178	5d .170	6d .199	7d .188	8d .186	9d .186
Co										
LT1day	+									
LT2day	+									
LT3day	+									
LT4day	+	+		+						
LT5day	+	+								
LT6day	+	+	+	+	+	+				
LT7day	+	+	+	+						
LT8day	+	+	+	+						
LT9day	+	+	+	+						

+: 95% significant

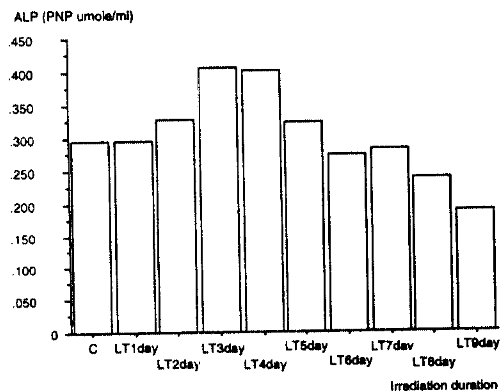

Figure 4. Histogram showing the differences in an amount of ALP of ROS according to the irradiation duration

contained MEM and F-12 subsequently for up to 9 days. Significant changes in ALP activity were observed when both osteoblastic cells were treated with LLLI. As seen in Table 3, 4 and Figure 3, LLLI has a stimulation effect on ALP activity of ROB with 17-54% magnitude when compared with control ($p<.0001$). Figure 4 shows the effect of LLLI on the ALP activity in culture of ROS17/2.8 cells. LLLI had a stimulation effect on the ALP activity of ROS17/2.8 cells with up to 37% magnitude when compared with control ($p<.0001$). In this study, ALP activity was enhanced maximally by LLLI in the 6-day irradiation group of ROB and in the 3-day irradiation group of ROS17/2.8. In addition, the ALP activity of ROB cells

Table 5. Means and standard deviations of ALP in all groups of ROS measured according to the irradiation duration

(PNP umoie/ml)

Group	Count	Mean	Std. Dev.
Co	5	.296	.046
LT1day	5	.295	.021
LT2day	5	.328	.072
LT3day	5	.406	.095
LT4day	5	.403	.090
LT5day	5	.324	.059
LT6day	5	.275	.038
LT7day	5	.283	.043
LT8day	5	.239	.049
LT9day	5	.188	.016
p value		<.0001	

Table 6. Results of multiple comparison test for all groups of ROS according to the irradiation duration

	Co .296	1d .295	2d .328	3d .406	4d .403	5d .324	6d .275	7d .283	8d .239	9d .188
Co										
LT1day										
LT2day										
LT3day	+	+	+							
LT4day	+	+	+							
LT5day				+	+					
LT6day				+	+					
LT7day				+	+					
LT8day			+	+	+	+				
LT9day	+	+	+	+	+	+	+	+		

+: 95% significant

Table 7. Means and standard deviations of ROS bone nodules measured after

Group	Count	Mean	Variance	Std. Dev.
Co	8	9.750	36.786	6.065
Ep	8	21.250	174.786	13.221
	P=.0421			

Table 8. Means and standard deviations of ROS bone nodules measured after LLLI

Group	Count	Mean	Std. Dev.
Co	12	24.250	11.910
Ep	12	38.500	13.514
	P=.0119		

in all groups was increased gradually by LLLI according to the irradiation duration, whereas that of ROS17/2.8 cells was decreased in the 6,7,8, and 9-day irradiation groups and rather inhibited significantly than in the control group.

Effect of LLLI on the calcified nodule formation of ROB and ROS17/2.8

To investigate the effect of LLLI on the calcified nodule formation, ROB and ROS17/2.8 cells were maintained up to 21 days with treatment of LLLI in experimental group. The number of calcified nodules was significantly increased in the irradiation group of both ROB and ROS cells after LLLI for 21 days (Table 7, 8).

Ⅳ. Discussion

Osteoblasts, primarily responsible for bone formation, produce Type I collagen and noncollagenous proteins as well as a wide variety of biomolecules which affect on the regulation of cell division, cell differentiation and bone mineralization. Alkaline phosphatase (ALP) and osteocalcin are classical marker for the osteoblastic phenotype.

In this study, the growth of ROB and ROS cell groups were slightly increased when compared with that of control group, but there was no significant difference between them. However, the formation of bone nodule and the ALP activity in the irradiation group was

significantly stimulated after LLLI compared with control group. In the experiment for the growth of cells LLL was irradiated for 1 minute every 12 hours during 11 days until the dish was completely covered with the cells and in the experiment for the bone nodule LLL was irradiated for 1 minute 3 times a week during 3 weeks respectively, while in the experiment of the ALP LLL was irradiated accumulatively according to the group (LT1day irradiation group; irradiated only at the 1st day of experiment, LT2day irradiation group; irradiated at the 1st and 2nd day of experiment, etc.). Therefore, the results from these experiment can not be compared with each other, but it is believed that under the optimal condition there was no significant difference in the growth rate of cells between the control and the irradiation groups*, but the bone formation was stimulated by LLLI regardless of cell growth. It is believed that the dish for the bone formation was not in the optimal condition because the experiment was performed being filled with cells in a dish. Steinlechner and Dyson reported that cells from taken from confluent cultures exhibited a decreased capacity for proliferation, the control showing very little proliferative activity in contrast the irradiated cultures all proliferated relatively rapidly. These results agree with them of Rigau動 study on the fibroblast that there was a definite increase in the overall proliferation and metabolism of LLLT-irradiated fibroblasts in vitro, although the actual number of fibroblasts remained unchanged.

In Figure 3 and 4, we can see that the ALP activities of ROB and ROS cells were peak in the 3 day irradiation group and in the 6 day irradiation group respectively. After being maximally increased, the ALP activities came to be stagnant or rather decreased significantly. These results mean that frequent irradiation of LLL on the cells has no biostimulation effect but rather an inhibitory effect on cell growth and an ideal interval of irradiation should be investigated for the promotion of cell growth.6

Although there have been several clinical reports that LLLI appears to stimulate bone formation, little is known about its possible role in osteoblastic function. In this study, we demonstrated that LLLI stimulates the alkaline phosphatase activity and calcified nodule formation. These results suggest that the LLLI is involved in the osteoblastic bone formation. Therefore, we may proposed that the LLLI may be applied under pathological conditions such as localized bone defect, skeletal fracture nonunion, and osteoporosis.

In addition osteoclasts and osteoblasts are closely related in the process of bone remodeling with a highly ordered sequence of bone resorption and formation, therefore it will be additionally helpful to study the LLLI effects on osteoclastic function as well whether LLLI regulates the activity of osteoclastic bone resorption.

It is unclear which mechanisms are involved in the stimulatory action of LLLI on ALP activity and calcified nodule formation in this experiment. However, the stimulatory action of LLLI in ALP activity and calcified nodule formation in this study seems to be associated, at least in part, with the osteoblastic bone formation, although the precise action of LLLT remains to be elucidated in detail

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국문초록

백서조골세포의 기능에 대한 저수준레이저의 효과

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저수준레이저조사(low level laser irradiation, LLLI)가 골형성을 자극한다고 보고되고 있지만 골아세포기능에 있어서 어떠한 역할을 하는지 별로 밝혀진 바가 없다. 본 연구에서는 백서 두개관 조골세포양 세포(ROB)와 백서 조골세포주(ROS17/2.8)에 대한 저수준레이저의 효과를 조사하기 위하여 세포증식, alkaline phosphatase activity, 석회화 결절형성등을 관찰하였다. 두가지 배양 세포군에 저수준레이저를 조사한 결과 저수준레이저가 alkaline phosphatase activity를 증가시킬 뿐만 아니라 석회화 결절의 형성을 촉진하는 것을 볼 수 있었다. 이러한 결과로 보아 저수준레이저는 조골세포의 기능과 무기질침착을 자극함으로써 골형성을 촉진한다고 추정된다.