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Effects of Low-level laser irradiation on the ALP activity and calcified nodule formation of rat osteoblastic cell

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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 Low Level Laser Irradiation (LLLI) had decreased 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 reactive-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 were increased with fibroblast proliferation at the lower energy density (2 J/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 biostimulation effects on all kinds of cells.

Various attempts have been made to enhance bone growth or healing of bone defects. These include autogenous bone grafting[12], application of various growth factors; mainly bone morpho-

genetic proteins,[13-16] use of low-intensity pulsed ultrasound[17], and use of electromagnetic fields.[18-21] However, the effect of electromagnetic irradiation such as low-energy laser irradiation on bone growth and repair has been investigated to a very limited extent. Abe[22] presented the application of LLLT for treatment of a fracture in an older patient with the added complication of chronic osteomyelitis of more than 20 years' standing and suggested that LLLT would be a non-invasive and effective tool to enhance bone fusion for the treatment of fractures, especially with added complications such as osteomyelitis. Trelles et al.[23] reported a faster formation of callus and vascularization in induced fractures in the mouse by 12 consecutive irradiations of He-Ne lasers, based on nonquantitative histological observations. Kusakari et al.[24] studied the effect of low-power laser irradiation(789 nm wavelength) on healing of focal injuries in the mandibular bone and in UMR 106 osteoblast-like cells in tissue cultures. They reported promotion of bone formation by laser irradiation based on histological examination, stimulation of DNA and protein synthesis and elevation of alkaline phosphatase (ALP) activity in the cells in culture. In recent study to investigate the influence of low level diode laser therapy (830 nm 30 mW C/W and 904 nm, pulsed, peak power, 30 nm)on tibial fracture healing using mice, it was reported that the effect of pulsed 904 nm irradiation was significantly higher than 830 nm C/W laser irradiation and the photoacoustic generation of an ultrasonic wave by the pulsed laser in the irradiated bone may play an additional role in the mechanism of callus formation.[25]

The purpose of this study is to investigate the effect of LLLI (904 nm of GaAs semiconductor laser) on the osteoblastic function such as ALP activity and calcified nodule formation to study whether 904 nm of GaAs laser is as effective as 630 nm of HeNe laser, and to establish the proper criteria for irradiating GaAs pulsed laser in clinical use.

II. Materials and Methods

Laser apparatus

BIOLASER(Dong Yang Medical, Korea), using GaAs semiconductor as the diode was used as the laser apparatus for this study. It is an infrared laser apparatus with a wave-length of 904nm. Peak output power is 27 W and average output power is 27 mW. Used pulses were pulse 1(P1, 5 Hz, 1 mW of average output power), pulse 7(P7, 500 Hz, 1mW of average output power), pulse 9(P9, 1,500 Hz, 3mW of average output power), pulse 11 (P11, 3,000Hz, 6mW of average output power), pulse 13 (P13, 6000 Hz, 14 mW of average output power) and pulse 15 (P15, 10,000 Hz, 27mW of average output power).

Laser irradiations

The optimum distance from the laser probe to the base of dish was determined with a laser beam detector, 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). LLLI were performed one time a day for 7 and 9 days to study the ALP formation respectively and 3 times a week (every other day irradiation) for 21 days to study a calcified nodule formation (Table 1,2 and 3).

Table 1. Laser irradiation schedule and total energy densities of all groups used in the 1st experiment

Group\Day	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Total energy density J/cm^2
Co (n=5)	-	-	-	-	-	-	-	
D1 (n=5)	+	-	-	-	-	-	-	1.313
D2 (n=5)	+	+	-	-	-	-	-	2.626
D3 (n=5)	+	+	+	-	-	-	-	3.939
D4 (n=5)	+	+	+	+	-	-	-	5.252
D5 (n=5)	+	+	+	+	+	-	-	6.565
D6 (n=5)	+	+	+	+	+	+	-	7.878
D7 (n=5)	+	+	+	+	+	+	+	9.191
De2(n=5)	+	-	+	-	+	-	+	3.939

Table 2. Group characteristics for the 2nd experiment

Group	Frequency (Hz)	Sample size	Energy density (J/cm2)	Irradiation duration (day) day	Total energy density (J/cm2)
Co	-	35	.0	9	.0
P1	5	10	.842	9	.421
P7	500	10	.842	9	.421
P9	1500	10	2.531	9	1.269
P11	3000	10	5.063	9	2.546
P13	6000	10	11.817	9	5.895
P15	10000	10	22.779	9	11.367

Table 3. Group characteristics for the 3rd experiment

Group	Duration (day)	Total energy (J/cm^2)	Sample size
Co	21	.0	12
P1	21	.421	6
P7	21	.421	6
P9	21	1.269	6
P11	21	2.546	6
P13	21	5.895	6
P15	21	11.367	6

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

Osteoblast-enriched cells were isolated from 19 day-old fetal Sprague-Dawley rat calvaria according to the methods described by Wong and Cohn.[26] Briefly, rat calvaria were dissected aseptically with enzyme solution containing 0.1% collagenase, 0.05% trypsin and 0.5 mM EDTA. At 10, 20, 30, 50 and 70 minutes after operation (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 ^{0}C in 95% humidified air containing 5% CO2. After 7 days, the cells were plated in culture dishes or culture plates according to its purposes.

Experiment for the effect of LLLI on the ALP formation according to the irradiation duration

To determine the effect of LLLI on the ALP activities of rat osteoblastic(ROB) cells according to the irradiation duration, ROB 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 w^{a}s obtained. Forty five samples for the effects of LLLT on the ALP activity according to the duration were randomly divided into 8 groups: control, D1(irradiated only on the 1st day), D2(irradiated on the 1st and 2nd day), D3, D4, D5, D6, D7(irradiated for 7 days), and De2(irradiated every other day) groups. The sample size of all groups was 5 respectively. To compare the ALP activities, the cells were irradiated with laser for 30 seconds every day for up to 7 days according to the group and the ALP activities were assayed simultaneously on the 8th day. 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 ^{0}C for 10 min. The optical density of p-nitrophenol, a reaction product, was read at 405nm using ELISA reader (SLT Labinstruments, 400 SFC).

Experiment for the effect of LLLI on the ALP activity according to the pulse type

To determine the effect of LLLI on the ALP activities of rat osteoblastic cells according to the pulse type, ROB 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 cultured until confluency was obtained. Ninty five samples for the effects of LLLT on the ALP activity according to the pulse type were randomly divided into 7 groups: control, P1, P7, P9, P11, P13, and P15 groups. The control group was 35 and the experimental groups were 10 respectively. To compare the ALP activities, the cells were irradiated with laser for 30 seconds every day for up to 9 days according to the group and the ALP activities were assayed simultaneously on the 10th day. 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 ^{0}C for 10 min. The optical density of p-nitrophenol, a

reaction product, was read at 405 nm using ELISA reader (SLT Labinstruments, 400 SFC).

Experiment for the effect of LLLI on the formation of calcified nodule

To determine the effects of LLLI on the calcified nodule formation through the long-term culture of osteoblastic cells, ROB 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 cultured until reached confluency. After confluency, the cells were fed with media supplemented with 50 mg/ml ascorbic acid and 10 mM glycerophosphate and LLLI was performed during the culture. Media were changed every second or third day and cultures were maintained for 21 days. Forty eight samples for the effects of LLLT on the formation of calcified nodule according to the pulse type were randomly divided into 7 groups: control, P1, P7, P9, P11, P13, and P15 groups. The control group was 12 and the experimental groups were 6 respectively. 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.

Statistical analysis

Statistical comparisons were made, using Statview 4.0 for Macintosh computor. To determine a significance of effects on the ALP and calcified bone formations of ROB after LLLI, ANOVA test was used. To compare the effects of LLLI according to the pulse type and the irradiation duration, Fisher's Protected Least Significant Difference (PLSD) test was used.

Ⅲ. Results

Effect of LLLI on the ALP formation according to the irradiation duration in the cultured ROB cells

To study the effects of LLLI on the ALP activity of ROB cells, cells were treated with LLLI for 30 min a day for different periods according to the group(Table 1). Table 4 shows the means and standard deviations of all groups and the results of ANOVA tests. LLLI increased slightly ALP formation with irradiation duration (Figure 1). There was, however, no significant difference statistically among the groups. In addition there was no significance statistically, but ALP formation was increased after LLLI everyday for 7 days.

Effects of LLLI on the ALP activity according to the pulse type in the cultured ROB cells

To determine whether LLLI affects on the ALP activity of ROB cells, the cells were treated with LLLI in 0.4% FBS contained MEM and F-12 subsequently for up to 9 days. Significant changes in ALP activity were observed when both osteoblastic cells were irradiated with P13 and P15 lasers. As seen in Table 5, 6 and Figure 2, LLLI has a stimulation effect on ALP formation of ROB with 65-74% magnitude in P13 and P15 groups when compared with control ($p<.05$). However, there was no significant difference between P13 and P15 after LLLI. In this study, ALP activity was enhanced in the total energy densities of 11.817 to 22.779 J/cm^2 by LLLI, however, it does not show a significant changes in P1, P7, P9 and P11 under 5.063 J/cm^2 of total energy density compared with control group.

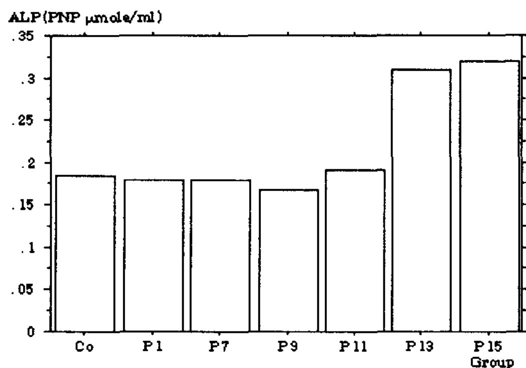

Figure 1. Histogram showing the changes of ALP actvity measured according to the irradiation duration

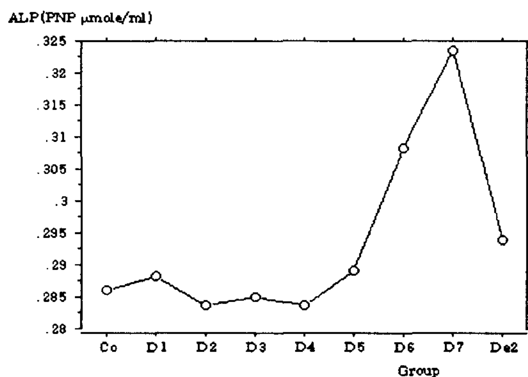

Figure 2. Linear graph showing the differences of ALP activity measured according to the pulse type

Table 4. Means and standard deviations of ALP activity in all groups of ROB measured according to the irradiation duration and results of ANOVA test

Group	n	Mean	Std. Dev.
Co	5	.286	.028
D1	5	.288	.016
D2	5	.284	.026
D3	5	.285	.032
D4	5	.284	.018
D5	5	.289	.02
D6	5	.308	.063
D7	5	.323	.059
De2	5	.297	.014
p value		-	

Table 5. Means and standard deviations of ALP activity in all groups of ROB measured according to the pulse type and results of ANOVA test

Group	Means	Std. Dev.
Co	.184	.042
P1	.180	.017
P7	.179	.021
P9	.167	.017
P11	.192	.040
P13	.309	.156
P15	.320	.142
p value	<.0001	

Table 6. Results of Multiple comparison test for ALP activity according to the pulse type

		Co	P1	P7	P9	P11	P13	P15
Co	.184							
P1	.180							
P7	.179							
P9	.167							
P11	.192							
P13	.309	+	+	+	+	+		
P15	.320	+	+	+	+	+		

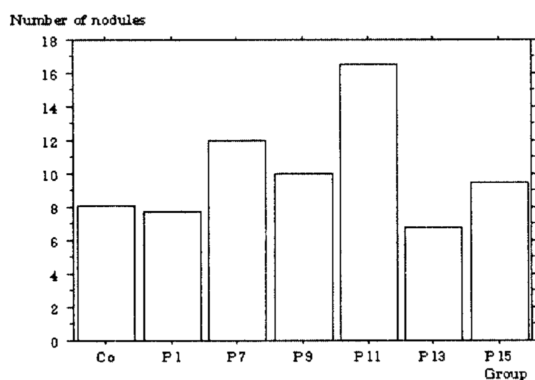

Figure 3. Histogram showing the differences of bone nodule formed according to the pulse type

Table 7. Means and standard deviations of calcified nodule measured according to the pulse type and results of ANOVA test

Group	n	Means	Std. Dev.
Co	8	8.1	3.9
P1	4	7.8	2.4
P7	4	12.0	4.8
P9	4	10.0	4.5
P11	4	16.5	1.3
P13	4	6.8	2.1
P15	4	9.5	1.7
p value		<.0055	

Effect of LLLI on the calcified nodule formation of ROB cells according to the pulse type

To investigate the effect of LLLI on the calcified nodule formation, ROB cells were maintained for 21 days with treatment of LLLI in experimental group. The number of calcified nodules was significantly increased in P11 group of ROB cells after LLLI for 21 days (Table 7, 8 and Figure 3), although there was no significant difference in P1, P7, P9, P14 and P15, compared with control group. In this study, the formation of calcified nodule was most stimulated in the total energy density of 0.861 J/cm^2, differently from the results of the 2nd experiment for ALP activity which LLLI had a stimulation effect on ALP formation of ROB in P13 and P15 groups when compared with control.

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 the classical markers for the osteobl-

Table 8. Results of Multiple comparison test for the formation of calcified nodule in all groups classified according to the pulse type

		Co	P1	P7	P9	P11	P13	P15
Co	8.1							
P1	7.8							
P7	12.0							
P9	10.0							
P11	16.5	+	+		+			
P13	6.8			+		+		
P15	9.5					+		

astic phenotype.[27-29]

Although the function of skeletal ALP in vivo is unknown, the enzyme is thought to be involved in bone formation,[30-32] and, consistent with that premise, several lines of evidence suggest that measurement of skeletal ALP activity in serum might provide a useful index of the bone formation rate: (1) skeletal ALP is localized in the plasma membranes of osteoblasts, (2) in vitro studies have shown that the amount of ALP activity in fetal rat calvaria is proportional to the rate of collagen production.[33] (3) in vivo studies with normal young mice have shown a correlation between serum ALP activity and osteoblast number,[34] (4) the amount of skeletal ALP activity in human serum is proportional to the rate of bone formation, at least when both are elevated, as in young children[35] and patients with PagetÕs disease.[36] In Farley and BaylinkÕs study ,[37] they suggested that, in the absence of skeletal effectors, measurement of calvarial alkaline phosphatase activity in vitro can serve as a useful index of calvarial collagen formation. These reports support skeletal ALP could be used as a predictive index of the capacity for in vitro collagen synthesis in this study.

In the 1st experiment of this study, the ALP activities were slightly increased when compared with that of control group, but there was no significant difference between them. In the previous preliminary study,[38] however, ALP activity was increased to approximately two folds of controls ($p<.0001$) in the 3rd and 4th day groups of the ROS cells and in the 5th and 6th day groups of the ROB cells. This can be explained that the photosensitivity of cells is not an all-or-nothing phoenomenon, but depends on various degrees on the physiological state of the cell before irradiation.[39] It was, however, suggested in the previous study 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]. In fact, the irradiation interval in that study was 12 hours and that in this study was 24 hours. This was the reason that the duration of 2nd experiment in this study should be extended upto 9 days because physiological condition before irradiation could not be controlled identically.

Those results in this study support that LLLT in the irradiated bone may play an additional role in the mechanism of callus formation,[40] a low energy level argon ion laser, not usually considered as an LLLT system, has a biostimulation effect on the bone regeneration,[41] LLLT controlled the inflammatory response to the hydroxyapatite implant resulting in better implant bonding and osteogenesis at implant borders,[42] and near infrared LLLT using diode lasers accelerates bone fracture healing.[43]

The most significance outcome of the present study is the demonstration that low-energy laser irradiation at proper time, frequency, and energy level can alter the activity of osteoblasts, as reflected by the change in ALP activity and bone nodule formation, and cause approximately 70 % and 100 % promotion of ALP activity and bone nodule formation respectively after LLLT in an vitro model.

About 70% more ALP activity in the experimental groups 9 days after irradiation as compared with control groups may indicate increase in ALP activity in these cells. One may assume that the significant promotion of the bone nodule formation in the laser-irradiated groups, as indicated by microscopical observation, is a result of increase in the photobioactivation of osteoblasts after LLLT. Barushka et al.[44] reported that significant promotion of the rate of repair in the laser-irradiated rats is a result of elevation in the number of active osteoblasts in the injured site

after stimulation. This study was, however, performed to investigate the change of ALP activity and bone nodule formation in the dishes cultured confluently with osteoblasts. This means that the faster healing of the hole injury with new reparative bone in the laser-irradiated rats could be done due to the biostimulation of osteoblast function itself increasing ALP activity as well as the increase of number of osteoblasts. It should be noted that it was proposed previously that laser irradiation may affect cells and process in different ways.[45,46] For example, after injury to the optic nerve, He-Ne laser irradiation delays the degeneration porcess,[47] whereas after injury to skeletal muscle, degeneration is enhanced by laser irradiation.[48] In addition, It was found previously the peculiar phenomenon that the positive effect of the laser irradiation may be achieved only remote from the irradiation period in generation of skeletal muscles.[49] The photosensitivity of cells is not an all-or-nothing phenomenon (as evidenced by the seasonal variations of the light-growth response), and the cell can respond to the light stimulus in various degree.[50] The magnitude of the photoresponse depends on the physiological state of the cell prior to irradiation which is conditioned by, for example, the amount of nutrients available and the age of the culture. Starving cells are more photosensitive than well-fed ones,[51] and the extent of light-inducible respiration varies with the degree of starvation.[52] Usually, cells in the exponential phase of growth are more photosensitive than those in the phase of stationary growth.[53] In this study, the results of 2nd experiment show that there was significant increase of ALP activity in the P13 and P15 group, the result of 3rd experiment, however, shows that there was a significant increase of number of bone nodule in the P11 group and there were, furthermore, no differences in the P13 and P15 groups compared with the control group although, in the 2nd experiment, P13 and P15 increased the ALP activity. These results are explained by KaruÕs report mentioned previously and the precise regulatory mechanisms of the stimulatory process by laser irradiation are not yet clear. It, therefore, should be noted that osteoblasts of short and long term experiments in this study could be stimulated differently.

The results of the present study using an experimental in vitro model indicate that the use of low level laser therapy to promote healing of fractures or defects in bones may prove promising in the future. However, the present study only establishes the possibility of using laser irradiation in vivo experiment or clinical situations. Further studies must define the energies and proper timing of irradiation in humans and its application to internal bone defects.

Although there have been several clinical reports that LLLI appears to stimulate bone formation,[54-58] little is known about its possible role in osteoblastic function and 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. 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.

V. Conclusions

The effect of low-level laser(GaAs) irradiation on the ALP activity and calcified nodule was investigated using biochemical and quantitative histological methods. The activity of alkaline phosphatase (ALP) showed a slow increase with irradiation duration (total energy under 5.28 J/cm^2) for 7 days of experiment using pulse 13 (p>.05). In the experiment to investigate the effect of LLLI according to the pulse type, the ALP activity showed a sharp peak in pulse 13 and 15 (total energy over 5.895 J/cm^2) significantly. The histological evaluation revealed a more rapid formation of calcified nodule in pulse 11 group (total energy 2.546 J/cm^2). It is concluded that GaAs semiconductor laser irradiation at proper energy probably affects the ALP activity and calcified nodule in the cultured osteoblast, as demonstrated by alteration in ALP activity and calcified nodule formation. These results also raise the possibility that LLLI could promote osteoblastic bone formation by stimulating osteoblastic function.

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국문초록

저수준레이저(GaAs 반도체)조사가 골모세포의 알칼리성 인산분해효소의 활성과 석회화결절의 형성에 미치는 영향

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저수준레에저요법에 대해서는 지난 10여년간 의학계 및 치과계에서 임상적으로 사용하여 좋은 결과가 있다고 많은 보고가 발표되고 있다. 특히 최근의 골결손에 관한 연구에서는 전기요법, 초음파요법, 전자장요법 등 뿐만아니라 저수준레이저를 사용하여 골절부내 callus형성이 촉진되었음을 보고하고 새로운 치료법의 하나가 될 수 있음을 제안한 바도 있다. 본 연구에서는 갈륨비소를 다이오드로 사용한 저수준레이저조사가 골결손의 치유에 어떠한 영향을 미치는 지 확인하고저 골모세포의 알칼리성 인산분해효소의 활성화와 석회화결절의 형성을 평가함으로 골모세포의 기능을 조사코저하였다. 실험은 첫째, 9개군으로 나누어 레이저 조사기간에 따른 알칼리성 인산분해효소의 활성화를 조사하였고, 둘째, 이를 근거로9일간 계속 매일 1회 1.3 J/cm^{2}의 레이저를 조사한 후 펄스의 종류별 차이를 비교하였으며, 세째, 레이저펄스별 석회화 결절의 형성 정도를 광학현미경으로 관찰하여 비교분석하였다. 결과, 7일 계속 레이저를 조사한 경우 다른 군에 비해 서서히 ALP의 활성이 증가하였으나 유의한 차이는 없었으며, 따라서 9일동안 레이저를 계속 조사한 경우에는 전체 에너지량이 5.895 J/cm^2 인 펄스13과 15가 뚜렷하게 유의한 증가를 보여주었다. 그러나 석회화결절의 형성은 전체 에너지량이 2.546 J/cm^2 인 펄스11에서 가장 많았다. 결론적으로 골형성이나 알칼리성 인산분해효소의 활성을 촉진하는 데에는 적절한 레이저 조사조건이 필요하나, 알칼리성 인산분해효소의 활성을 촉진한 펄스와 석회화결절의 형성을 촉진하는 펄스가 서로 다르게 나타난 것은 골형성을 촉진하는 여러요인 들이 저수준레이저에 자극받았을 가능성이 높음을 보여준다. 이러한 결과들로 보아 저수준레이저는 골모세포의 기능을 자극하여 골결손의 치유를 개선하는 데 도움될 것이라 사료된다.