Effects of low reactive level laser irradiation (LLLI) on the wound infected with *Staphylococcus aureus*

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Contents

I. Introduction

LASER is an acronym stands for Light Amplication by the Stimulated Emission of Radiation. Einstein provided the basic theory of the laser in 1917. Maiman, who was a physicist at Hughes Research Laboratories, produced a ruby-laser which became the first true laser.[1] Laser light is transmitted through tissue, reflected from it, scattered within or absorbed by tissue. When a high power laser beam strikes target tissue, it is absorbed and causes a sharp rise in temperature of the irradiated tissue. The tissue structure is either destroyed or permanently altered.

On the other hand,scattered or transmitted beam of low power laser is ultimately absorbed in the cellular level and produce non thermal effects; no alteration in tissue structure and reaction below the destructive threshold. This reaction can be referred to as Low reactive-level laser irradiation(LLLI). LLLI is a new, internationally accepted name for biostimulation by low energy lasers in order to achieve therapeutic desired effects and a reversible form of laser therapy without tissue destruction. A popular benchmark for defining a LLLI system is an output power of less than 100 mW. LLLI is commonly regarded as safe for the irradiation of skin.[2]

Various studies on low reactive level laser (LLL) have been performed to investigate the therapeutic benefits of laser irradiation on wounds or lesions. Mester et al.[3] reported that the healing of total skin defects that artificially created on the back of white mice was significantly accelerated by the irradiation of ruby laser. Mester hypothesized that the beneficial interference of laser energy is related to an activation of the enzymes that was essential in the healing process. This was based on his observation of the beneficial effect of laser irradiation on the various stages of healing in 32 patients suffering from a variety of skin

lesions. Palmieri[4] reported that an increased activity of myofibroblasts might also be responsible for a quicker closure of the wound.

Chavrier et al.[5] also stated that the infrared GaAs laser has biostimulation effect. They proposed that the LLL irradiation may stimulate the protein and DNA synthesis to accelerate the proliferation of gingival fibroblast. The interest for laser therapy applied in healing of post-traumatic lesions and myopathies has recently increased for the investigation of the molecular basis for explaining therapeutic effects of LLL.[6-9]

The treatment of different pathological conditions with LLL irradiation has also been interested; LLLI has shown positive therapeutic effects on recurrent aphthous stomatitis acute herpetic stomatitis, exudative erythema multiforme and gingivitis.[10]

In 1987, Lee and Kim[11] reported that LLLI was effective in reducing gingival inflammation. They compared the irradiated and non-irradiated gingiva microscopically and microbiologically and concluded that the irradiated gingiva and the ratio of motile rods and spirochetes in the gingival sulcus were significantly decreased. They also suggested that LLLI have biostimulation effects on the growth of bacterial cells as well as tissue cells.

Kim et al. confirmed that LLLI also have the biostimulation effect on the growth of *Streptococcus mutans*,[12] *Candida albicans*[13], in the specific irradiation dose and time. The authors, therefore, concluded that LLLI has the biostimulation effects on the microorganisms as well as the tissue cells.

Lee et al.[14] reported an animal study in which LLLI is applied in an infected wound. It was suggested that the acceleration healing of infected wound following LLLI indicates that the cellular activity due to the biostimulation effect of LLLI in the surrounding normal tissue predominates over the tissue irritation due to the bacterial growth in the infected wound.

The purpose of this study was to compare the effect of central and peripheral irradiation for the wound infected with *S. aureus*. It was hypothesized that the promotion of healing in the infected wound following LLLI is due to an increase in the cellular activity via the biostimulation of LLLI in the surrounding normal tissue as well as the tissue irritation via an increase in the bacterial growth in the infected wound.

II. Materials and Methods

Materials

Laser Apparatus

The laser used in this work was the BIOLASER(Dong Yang Medical, Seoul, Korea) using GaAs semiconductor as a diode. It is a pulsed infrared laser apparatus with a wavelength of 904nm and peak output power is 27 W. Pulsed lasers used in this study were pulse 7 (500 Hz, 1 mW of average output power), pulse 9 (1500 Hz, 3 mW of average output power) pulse 11 (P11, 3000Hz, 6mW average output power), pulse 13 (P13, 6000Hz, 14mW average output power) and pulse 15 (10000 Hz, 27 mW of average output power).

Microorganism

Staphylococcus aureus used in this study were obtained from Department of Clinical Pathology, Shoonchunhyang Cheon-An Hospital, Choongnam Korea. The cells were activated two times before this experiment was done.

Culture medium

Culture medium (BHI) was prepared with

calf brains infusion from 200g, beef heart infusion from 250g, proteose peptone 10g, bacto dextrose 2g, sodium chloride 5g, disodium phosphate 2.5g in 1000ml of distilled water (pH 7.0). A single colony of *S. aureus* developed on the plate of the stock culture medium was transferred to 20 ml of the seed culture medium and incubated in a rotary shaking incubator at 25. The seed culture was inoculated into 100 ml of the medium. Temperature was controlled to 25 and culture pH was maintained to 7.0.

Animal

Healthy Sprague-Dawley female rats at approximately the same stage of oestrus weighing 250 to 300g were used.

Culture flask

Special flasks were made to allow repeated consistent measurement of the optical densities(OD). A pyrex tube (20ml) was attached to the neck of a standard Erlenmeyer flask (300ml) at an angle of about 25 as show in schematically in Figure 1.

Spectrophotometer

To evaluate the growth of *S. aureus*, spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, NY) was used to measure the optical density of cell culture broth. The spectrophotometer was set at 600nm. Reference liquid was identical brain heart broth media without cells.

Measuring Apparatus for wound contraction size

Reflector projector(Reflecta Reflecta GmbH Co., Germany) was used to trace the wound size and Planimeter(Keuffel & Esser Co. Germany) was used to measure the wound area traced in the tracing paper.

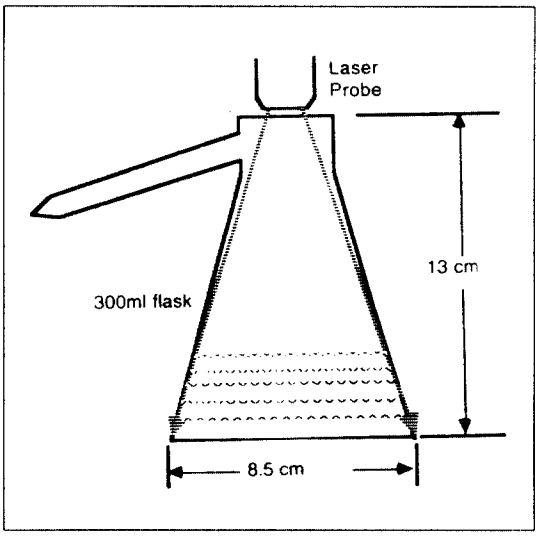

Figure 1: Schematic illustration of the relationship between the laser probe and the cell culture flask.

Methods

A variety of studies were performed to investigate the most proliferative pulse for *S. aureus* and to compare the effects of central and peripheral irradiation for the infected wound healing. These studies were categorized into two parts; Part I on the proliferation of *S. aureus* and Part II on the healing of infected wound.

Part I: Effect of LLL irraidation on the proliferation of S. aureus.

1. Experiment for the proliferation of S. aureus according to the pulse types

Twelve *S. aureus* cultures were used in this experiment. The cultures were randomly divided into 6 groups and coded according to the pulsing frequency: P7 (500Hz), P9 (1.5kHz), P11 (3kHz), P13 (6kHz), P15 (10kHz), and shame -irradiated control (Co) groups. The probe was

fixed vertically on a stand about 13 cm distant from the middle position of the base of the flask (Figure 1). After the inoculation of the seed culture, samples were irradiated for 1 minute at the beginning of experiment and then every 4 hours for 48 hours of cell growth and the optical density was measured using the spectrophotometer at the same time interval. All samples were cultivated in a shaking incubator (100 rpm) at 30 in the dark.

Energy densities of all experimental groups for each irradiation, P7, P9, P11, P13, and P15 were 1.06, 3.17, 6.35, 14.81 and 28.56 mJ/cm2, respectively.

2. Experiment for the proliferation of *S. aureus* according to the interval of irradiation

This experiment was performed to investigate the effect of LLLI according to the interval using the most proliferative pulse (P13) proved in the 1st experiment. Twelve samples were used in this experiment. The samples were randomly divided into 6 groups and coded according to the irradiation interval and the irradiation time: Hr2-1m (2 hour interval and 1 minute irradiation), Hr4-2m (4 hour interval and 2 minute irradiation), Hr6- 3m (6 hour interval and 3 minute irradiation), Hr8-4m (8 hour interval and 4 minute irradiation), Hr12-6m (12 hour interval and 6 minute irradiation), and Co (control) groups. After the inoculation of the seed culture, samples were irradiated from the beginning of experiment for 48 hours of cell growth according to the irradiation interval and time. OD was measured using the spectrophotometer every 4 hours. Energy densities of samples for each irradiation, Hr2-1m, Hr4- 2m, Hr6-3m, Hr8-4m, and Hr12-6m, were 14.81, 29.62, 44.43, 59.24, and 88.86 mJ/cm^2 respectively, but total energies of groups for 48 hours of the experiment were same, 355.44 mJ/cm^2.

3. Experiment for the proliferation of *S. aureus* according to the irradiation times

This experiment was performed to investigate the effect of LLLI according to irradiation time on the proliferation of *S. aureus*. The pulse and the interval used in this experiment was P13 (6000Hz, 14mW average output power) and 4 hours proved as the most proliferative pulse and the most effective interval for *S. aureus* in the previous experiment.

The samples were randomly divided into 5 groups and coded according to the irradiation time: Hr4-1m (4 hour interval and 1 minute irradiation), Hr4-2m (2 minute irradiatin), Hr4-3m (3 minute irradiation), Hr4-4m (4 minute irradiation), and Co (control) groups. After the inoculation of the seed culture, samples were irradiated from the beginning of experiment for 48 hours of cell growth according to the irradiation time. OD was measured every 4 hours. Energy densities of samples for each irradiation, Hr4-1m, Hr4-2m, Hr4-3m, and Hr4-4m, were 14.81, 29.62, 44.43, and 59.24 mJ/cm2 respectively.

Part II: Effect of central and peripheral irradiation of LLL for the wound infected with *S. aureus*

1. Experiment on the healing effect of peripheral LLLI for the infected wound

The animals were intramuscularly anesthetized with 1 mg/100 g of Ketalar (Ketamin HCl, 10 mg/ml, Parke-Davis & Co, U.S.A.) and 0.3 mg/100 g of Rompun (Xylazine, 20 mg/ml, Bayer, Korea). Following anesthesia, both left and right gluteal regions of all rats were shaved and cleaned with 75% alcohol and then

operation was performed aseptically .

The round, full-thickness skin defects measuring about 6 mm of diameter were produced on the gluteus superficialis of both hind limb in each rat. *Staphylococcus aureus* were cultured at 25℃ for 48 hours on BHI slant and suspended with 1ml of BHI broth, and then 5 ml of bacterial suspension was inoculated on the wounds. All wounds were left open without medication. The animals were kept in special wire mesh cages without bedding, so that the wounds remained, though not strictly sterile, in very clean conditions.

The laser irradiation and photograpy were also performed under anesthesia with Ketamin HCl and Xylazine. The probe of laser was placed vertically about 5 mm apart from the margin of the wound, and then laser was irradiated to the adjacent normal tissue around the wound, not to the wound, for 1 minute. Total area irradiated was about 3.3 cm. Energy density of experimental group was approximately 262.5 mJ/cm2. (Figure 2).

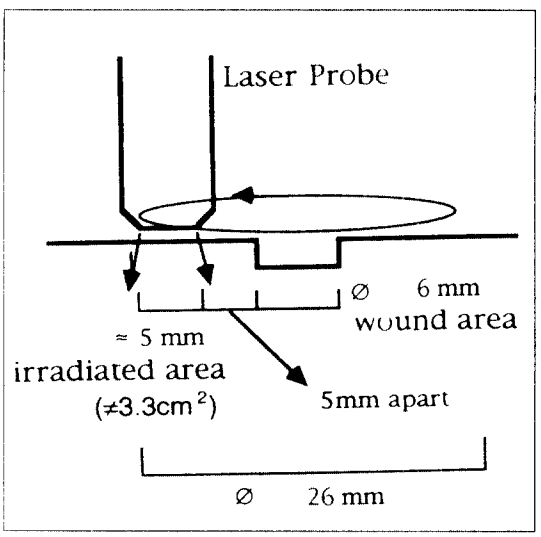

Figure 2: An illustration showing the peripheral application of the laser probe

Experimental wounds were irradiated on the 1st, 3rd, and 5th day, the first exposure being performed immediately after the operation and bacterial inoculation. Another wound was treated similary to experimental wound without LLL irradiation: they served as contralateral controls. The wounds were examined every other day and the clinical observations were recorded in a protocol. The wounds areas were determined by photographing them from a constant distance (via a constant objective focus) on the 1st, 3rd, 5th, and 7th postoperative days. The slides were projected through a reflector projector and the wound areas were traced, and then measured by planimeter. In each wound, the wound areas on consecutive days were expressed as a percentage of their initial area (on the first day). Twelve animals were used in this study but 4 were died during the experiment with unknown reason.

2. Experiment on the healing effect of central and peripheral LLL irradiation for the infected wound

The operation for the wound formation and the inoculation of *S. aureus* were performed identically with the 1st experiment. All wounds were left open without medication. The animals were kept in special wire mesh cages without bedding, so that the wounds remained, though not strictly sterile, in very clean conditions.

The laser irradiation and photograpy were also performed under anesthesia with Ketamin HCl and Xylazine. For one of both wounds, the probe of laser was placed vertically about 5 mm apart from the margin of the wound, and then laser was irradiated to the adjacent normal tissue around the wound, not to the wound, for 1 minute (Figure 2). For the other

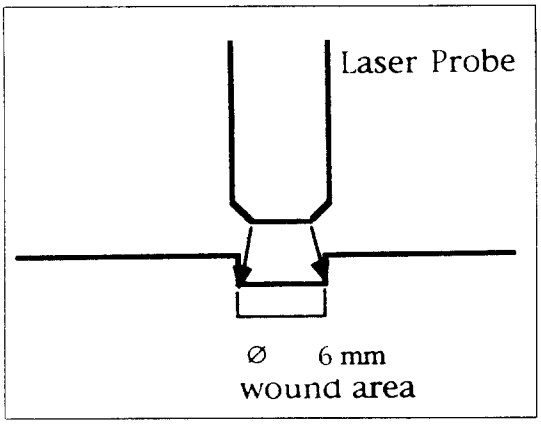

Figure 3: An illustration showing the contral application of the laser probe

wound of contralateral side, the probe was placed vertically about 1 mm above the wound and LLL was irradiated directly to the wound for 1 minute (Figure 3). Total areas irradiated centrally and peripherally were about 0.28cm^2 and 3.3cm^2 and energy densities of centrally and peripherally groups were approximately 3,000 mJ/cm^2 and 262.5 mJ/cm^2 respectively. (Figure 2).

LLL were irradiated on the 1st, 3rd, and 5th day; the first exposure being performed immediately after the operation and bacterial inoculation. The wound contraction size was also measured identically with the 1st experiment. Twenty subjects were used in this study.

3. Experiment on the healing effect of central LLL irraidation using the most proliferative pulse and peripheral LLL irradiation using the least proliferative pulse

The operation for the wound formation and the inoculation of *S. aureus* were performed identically with the 1st experiment. All wounds were left open without medication. The animals were kept in special wire mesh cages without bedding, so that the wounds remained, though not strictly sterile, in very clean conditions.

The laser irradiation and photograpy were also performed under anesthesia with Ketamin HCl and Xylazine. The probe was placed vertically about 1 mm above the wound and P7 was irradiated directly to the one and P13 to the other of both wounds for 1 minute (Figure 3). Energy densities of P7 and P13 group were approximately 214.29 and 3,000.0 mJ/cm^2.

LLLI were irradiated on the 1st, 3rd, and 5th day, the first exposure being performed immediately after the operation and bacterial inoculation. The wound contraction size was also measured identically with the 1st experiment. Twelve subjects were used in this study but one was died during the experiment with unknown reason

Statistical Analysis

All measurements in each group were averaged. Statistical comparisons were then made to determine the significance of the differences among the groups. Repeated measures ANOVA and Fisher's Protected Least Significant Difference (PLSD) were used.

Ⅲ. Results

Part I: Effect of LLL irraidation on the proliferation of S. aureus.

The means of all groups classified according to the pulse type and elapsed time are given in Table 1, and the changes of OD in all groups are shown in Figure 4. The results were then statistically tested whether the pattern of change over time is the same for the different pulse types by using repeated measures ANOVA. From the ANOVA test in Table 2, it is seen that pulse type exerted a

Table 1: Optical densities measured by the pulse type and the elapsed time during the incubation period of S. aureus. (n=2 per group)

group / incubation time	Co	P15	P13	P11	P9	P7
Hr0	.01±.00	.01±.00	.01±.00	.01±.00	.01±.00	.01±.00
Hr4	.02±.00	.02±.00	.02±.00	.01±.00	.02±.00	.01±.00
Hr8	.04±.00	.04±.00	.05±.00	.03±.00	.03±.00	.01±.00
Hr12	.09±.00	.08±.00	.11±.00	.05±.00	.06±.00	.03±.00
Hr16	.12±.01	.10±.01	.22±.00	.07±.00	.09±.00	.05±.01
Hr20	.17±.01	.15±.01	.35±.00	.10±.00	.12±.00	.07±.01
Hr24	.34±.01	.30±.01	.58±.00	.20±.00	.20±.01	.12±.01
Hr28	.55±.00	.48±.02	.72±.00	.32±.01	.34±.01	.19±.00
Hr32	.65±.00	.61±.01	.85±.00	.51±.01	.55±.01	.40±.00
Hr36	.79±.00	.76±.01	.92±.01	.62±.01	.68±.00	.53±.00
Hr40	.89±.01	.85±.00	1.00±.00	.75±.01	.78±.01	.69±.01
Hr44	.98±.00	.95±.00	1.10±.00	.90±.00	.90±.01	.80±.00
Hr48	1.10±.00	1.00±.00	1.20±.00	1.00±.00	1.10±.00	.85±.00

(Co, control ; P7,500Hz, 1mW ; P9, 1.5kHz, 3mW ; P11, 3kHz, 6mW ; 13, 6kHz, 14mW ; P15, 10kHz, 27mW)

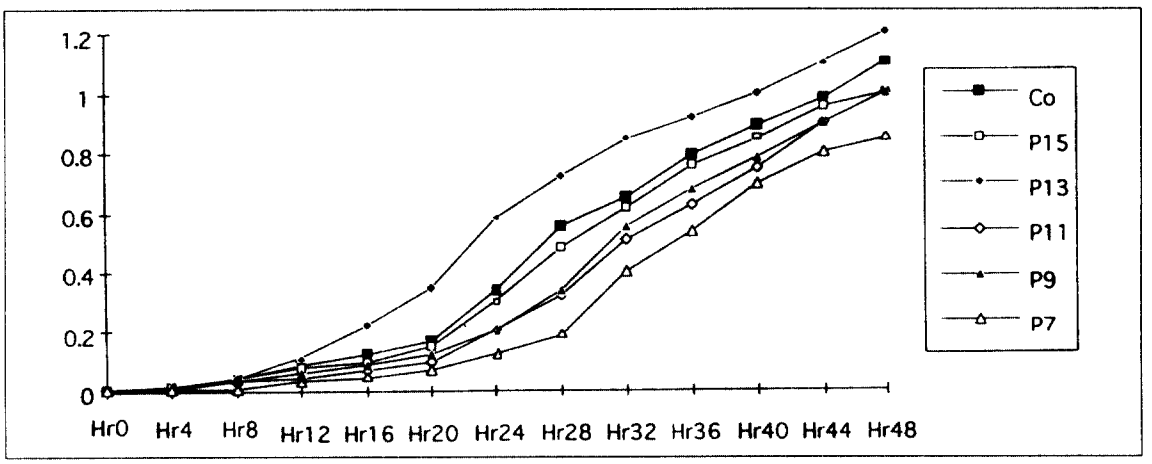

Figure 4: Linear graph showing the changes in the growth of S. aureus after LLLI (as measured spectrophotometrically by the optical density of the medium) as a factor of each pulse type and post-irradiation period, compared with the control medium.

Table 2: Results of ANOVA test for all optical densities measured according to the pulse type and time

	DF	Sun of Square	Mean Square	F-Value	P-Value
Pulse	5	1.02	0.20	586.0	<.0001
Subject(Group)	6	2.096E-3	3.494E-4		
Category for time	12	20.39	1.7	16201.42	<.0001
Category for time*pulse	60	0.52	8.70E-3	82.88	<.0001

highly significant influence on the cell growth ($p<.0001$). The interaction of pulse type and time also appears to have a highly significant influence ($p<.0001$). Table 3 shows the results of the multiple comparison test(Fisher's PLSD) for all of the groups and post-irradiation times. Highly significant differences between any groups paired were also noted from this test ($p<.0001$). P13 caused the most biostimulative effect on the proliferation of S. aureus as indicated in Figure 4, however, P7 decreased the growth of *S. aureus* more than any other pulses.

Table 3: Results of multiple comparison test (Fisher's PLSD) for all groups according to the pulse type.

	Mean Diff.	Crit. Diff	P-value
Co,P15	0.31	0.013	0.001
Co,P13	-0.105	0.013	<0.001
Co,P11	0.091	0.013	<0.001
Co,P9	0.075	0.013	<0.001
Co,P7	0.153	0.013	<0.001
P15,P13	-0.137	0.013	<0.001
P15,P11	0.06	0.013	<0.001
P15,P9	0.043	0.013	<0.001
P15,P7	0.122	0.013	<0.001
P13,P11	0.196	0.013	<0.001
P13,P9	0.18	0.013	<0.001
P13,P7	0.258	0.013	<0.001
P11,P9	-0.016	0.013	<0.001
P11,P7	0.062	0.013	<0.001
P9,P7	0.078	0.013	<0.001

Optical densities of all groups classified according to the irradiation interval at different incubation period of *S. aureus* were summarized in Table 4. The results of repeated measures ANOVA test according to the irradiation interval and the elapsed time are shown in Table 5. From the ANOVA test in Table 5, it can be seen that irradiation interval of LLL has a highly significant influence on the cell growth of *S. aureus* ($p<.0001$). The irradiation interval and time also were interacted significantly. Table 6 shows the results of the multiple comparison test (Fisher's PLSD) for all of the groups and post-irradiation times. Highly significant differences among all groups were

Table 4: Optical densities measured after LLLI with P13 according to the irradiation interval and the elapsed time during the incubation period of S. aureus. (n=2 per group)

group / incubation time	Co	Hr2-1min	Hr4-2min	Hr6-3min	Hr8-4min	Hr12-6min
Hr0	.01±.00	.01±.00	.01±.00	.01±.00	.01±.00	.01±.00
Hr4	.02±.00	.02±.00	.03±.00	.02±.00	.02±.00	.02±.00
Hr8	.04±.00	.05±.00	.07±.01	.03±.00	.04±.00	.04±.00
Hr12	.08±.00	.10±.00	.14±.00	.06±.00	.07±.00	.07±.00
Hr16	.15±.01	.20±.01	.28±.00	.12±.01	.13±.01	.16±.01
Hr20	.25±.01	.32±.00	.54±.00	.20±.00	.20±.01	.20±.01
Hr24	.38±.00	.40±.00	.67±.01	.30±.01	.29±.01	.30±.01
Hr28	.44±.00	.53±.00	.75±.00	.40±.00	.37±.01	.42±.00
Hr32	.59±.01	.62±.00	.88±.00	.55±.01	.50±.01	.50±.00
Hr36	.72±.03	.75±.00	.95±.01	.68±.01	.61±.00	.63±.00
Hr40	.85±.01	.85±.00	1.00±.00	.79±.01	.70±.01	.71±.01
Hr44	.98±.00	.98±.01	1.10±.00	.92±.01	.80±.01	.89±.00
Hr48	1.00±.00	1.00±.00	1.20±.00	1.00±.00	.85±.00	.94±.00

(Co, control ; Hr2-1min, 2 hour interval, 1min irradiation ; Hr4-2min, 4 hour interval, 2min irradiation ; Hr6-3min, 6 hour interval, 3min irradiation ; Hr8-4min, 8 hour interval, 4min irradiation ; Hr12-6min, 12 hour interval, 6min irradiation)

Table 5: Results of ANOVA test for all optical densities measured after LLLI with P13 according to the irradiation interval and the elapsed time.

	DF	Sun of Square	Mean Square	F-Value	P-Value
Group	5	0.93	0.18	3600.	<.0001
Category for time	12	18.65	1.55	29935	<.0001
Group+Category for time	60	0.43	7.217E-3	138.9	<.0001
Residual	78	4.050-3	5.192E-5		

Table 6: Results of multiple comparison test (Fisher's PLSD) for all the measurements of groups according to the irradiation interval.

	Mean Diff.	Crit. Diff	P-value
Co,Hr2-1min	-0.025	3.979E-3	0.001
Co,Hr4-2min	-0.161	3.979E-3	<0.001
Co,Hr6-3min	0.035	3.979E-3	<0.001
Co,Hr8-4min	0.075	3.979E-3	<0.001
Co,Hr12-6min	0.052	3.979E-3	<0.001
Hr2-1min,Hr4-2min	-0.137	3.979E-3	<0.001
Hr2-1min,Hr6-3min	0.06	3.979E-3	<0.001
Hr2-1min,Hr8-4min	0.099	3.979E-3	<0.001
Hr2-1min,Hr12-6min	0.076	3.979E-3	<0.001
Hr4-2min,Hr6-3min	0.197	3.979E-3	<0.001
Hr4-2min,Hr8-4min	0.236	3.979E-3	<0.001
Hr4-2min,Hr12-6min	0.213	3.979E-3	<0.001
Hr6-3min,Hr8-4min	0.039	3.979E-3	<0.001
Hr6-3min,Hr12-6min	0.016	3.979E-3	<0.001
Hr8-4min,Hr12-6min	-0.023	3.979E-3	<0.001

also noted from this test ($p<.0001$). As illustrated in Figure 5, the proliferation rate of Hr4-2min group was significantly higher than those of any other groups. This means that, under the identical condition of 29.62 mJ/ cm^2, 4 hour interval irradiation with P13 is the most effective in promoting the growth of S. aureus.

The means of optical densities measured after LLLI with P13 by the irradiation time and the elapsed time during the incubation period of *S. aureus* are given in Table 7. Table 8 is the results of repeated measures ANOVA test according to the irradiation time and the elapsed time. From the ANOVA test in Table 8, it is appearent that irradiation time has a highly significant influence on the cell growth of *S. aureus* ($p<.0001$). Table 9 shows the results of

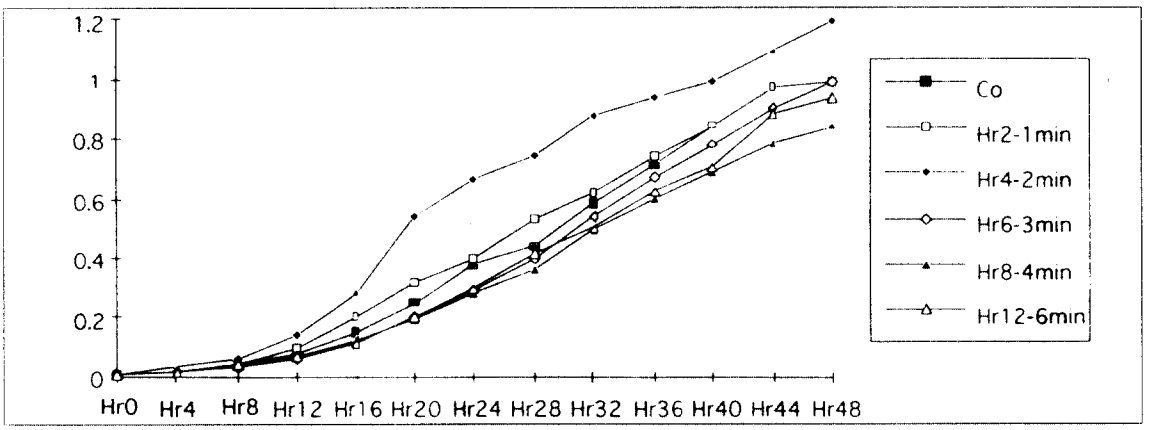

Figure 5: Linear graph showing the changes in the growth of S. aureus (as measured spectrophotometrically by the optical density of the medium) as a factor of each irradiation interval and post-irradiation period.

Table 7: Optical densities measured after LLLI with P13 according to the irradiation time and the elapsed time during the cell cycle of S. aureus. (n=2 per group)

group / incubation time	Co	Hr4-1min	Hr4-2min	Hr4-3min	Hr4-4min
Hr0	.01±.00	.01±.00	.01±.00	.01±.00	.01 ±.00
Hr4	.02±.00	.02±.00	.03±.00	.02±.00	.02 ±.00
Hr8	.05±.00	.06±.00	.08±.00	.07±.00	.05 ±.00
Hr12	.10±.00	.12±.00	.20±.00	.15±.00	.10 ±.00
Hr16	.15±.01	.18±.01	.35±.00	.19±.00	.19 ±.00
Hr20	.30±.01	.30±.01	.48±.00	.36±.00	.32 ±.00
Hr24	.40±.01	.41±.01	.59±.00	.48±.00	.44 ±.01
Hr28	.54±.00	.52±.02	.70±.00	.55±.01	.50 ±.01
Hr32	.66±.00	.60±.01	.84±.00	.65±.01	.60 ±.01
Hr36	.75±.00	.72±.01	.95±.01	.75±.01	.70 ±.00
Hr40	.88±.01	.83±.00	1.00±.00	.85±.01	.810±.01
Hr44	.95±.00	.98±.00	1.10±.00	.96±.00	.90 ±.01
Hr48	1.00±.00	1.10±.00	1.20±.00	1.00±.00	98 ±.00

(Co, control ; Hr4-1min, 4 hour interval, 1min irradiation ; Hr4-2min, 4 hour interval, 2min irradiation ; Hr4-3min, 4 hour interval, 3min irradiation ; Hr4-4min, 4 hour interval, 4min irradiation)

Table 8: Results of ANOVA test for all optical densities measured after LLLI with P13 according to the irradiation time and the elapsed time.

	DF	Sun of Square	Mean Square	F-Value	P-Value
Puls	4	0.36	0.09	2296.1	<.0001
Subject(Group)	5	1.96E-4	3.92E-5		
Category for time	12	16.79	1.39	79666.5	<.0001
Category for time+pulse	48	148	3.09E-3	175.7	<.0001
Category for time+Subject	60	1.05E-3	1.76E-4		

Table 9: Results of multiple comparison test (Fisher's PLSD) for all groups.

	Mean Diff.	Crit. Diff	P-value
Co,Hr4-1min	-6.923E-3	4.466E-3	0.0105
Co,Hr4-2min	-1.31	4.466E-3	<.0001
Co,Hr4-3min	-0.016	4.466E-3	0.0002
Co,Hr4-4min	0.015	4.466E-3	0.0003
Hr4-1min,Hr4-2min	-0.124	4.466E-3	<.0001
Hr4-1min,Hr4-3min	-9.231E-3	4.466E-3	0.0032
Hr4-1min,Hr4-4min	0.022	4.466E-3	<.0001
Hr4-2min,Hr4-3min	0.115	4.466E-3	<.0001
Hr4-2min,Hr4-4min	0.146	4.466E-3	<.0001
Hr4-3min,Hr4-4min	0.031	4.466E-3	<.0001

the multiple comparison test for all of the groups and post irradiation times. Statistically significant differences were indicated among the groups ($p<.05$). In the interaction line plot shown in Figure 6 it can be seen that the 2 min irradiation group is higher than other groups. This confirms that the irradiation time has a significant effect.

Part II: Effect of LLLI for the wound infected with S. aureus

The means of wound contraction rates mea-

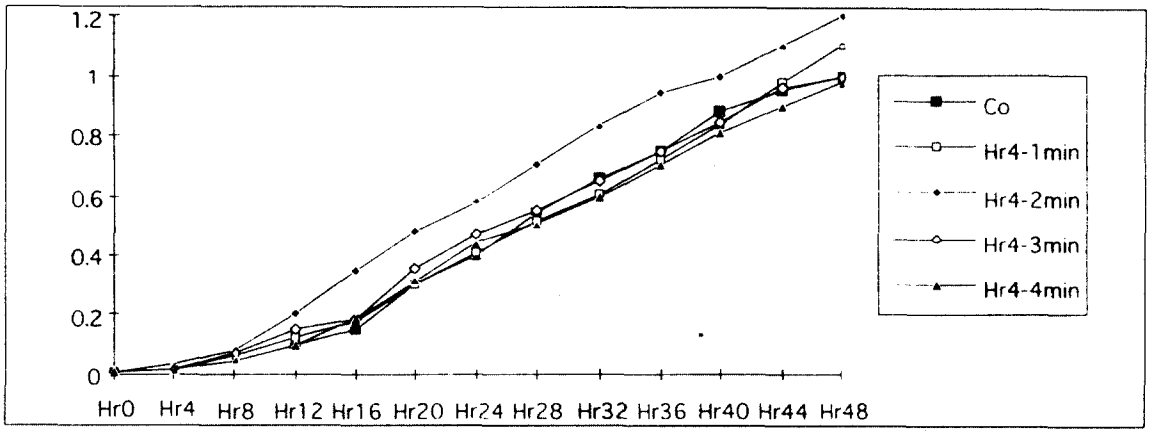

Figure 6: Linear graph showing the changes in the growth of S. aureus after LLLI with P13 (as measured spectrophotometrically by the optical density of the medium) as a factor of each irradiation interval and post-irradiation period.

sured in two groups, control group and peripheral irradiation group using P13 for 1 minute (14.81 mJ/cm^2), at the 1st, 3rd, 5th, and 7th day are given in Table 10. The results of repeated measures ANOVA test according to the groups and the elapsed time are given in Table 11

Table 10: The wound contraction rates(%) measured after LLLI (P13, 1min) according to the groups and the elapsed time. (n=8)

group	Time(day) of measurement			
	1st	3rd	5th	7th
Co	100.0±0	42.5±19.2	26.7±14.1	18.4±7.4
Pl	100.0±0	25.8± 6.9	15.8± 6.3	10.2±4.1

(Co, control group ; Pl, peripheral irradiation group)

and Figure 7. The between-group effect for peripheral irradiation is significant. This means that there is a significant difference in the effectiveness of the peripheral irradiation as measured by the wound contraction rate. From Figure 7, it is seen that the peripheral irradiation-by-time interaction arose from the fact that the peripheral irradiation group decreased significantly more at day 3 and 7.

The means of wound contraction rates measured according to the irradiation method, central and peripheral irradiation using the most proliferative pulse (P13), and the elapsed time are given in Table 12 and Figure 8. The results of repeated measures ANOVA test according to the groups and the elapsed time are given Table 13. The between-group main

Table 11: Results of ANOVA test for all rates of wound contraction measured after LLLI (P13, 1min) in control and peripheral irradiation groups with time.

	DF	Sun of Square	Mean Square	F-Value	P-Value
Type	1	1288.7	1288.7	5.89	0.029
Subject(Group)	14	3063.37	218.81		
Category for time	3	73986.74	24662.2	518.9	<.0001
Category for time*Type	3	577.08	192.36	4.04	0.012
Category for time*Subject	42	1995.91	47.52		

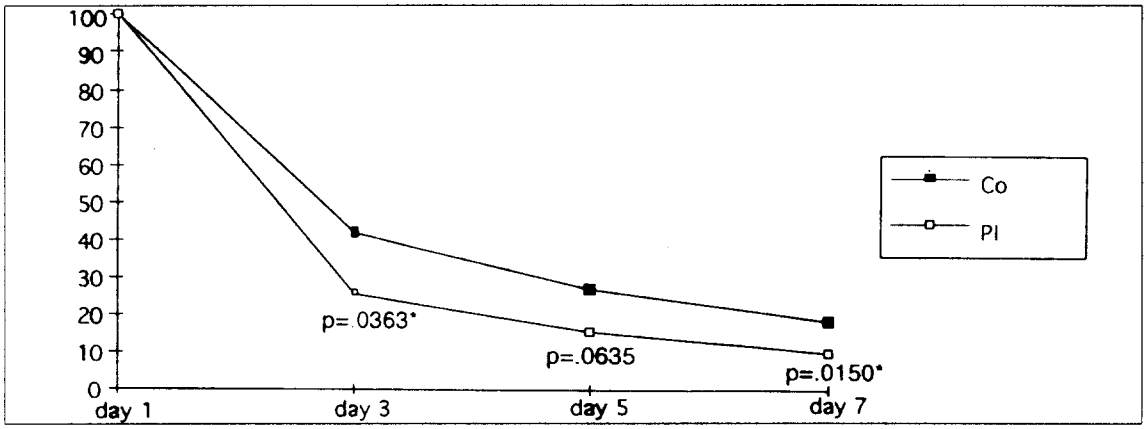

Figure 7: Linear graph showing the changes in the wound contraction rate as a factor of peripheral irradiation group (PI, P13, 1min) and post-irradiation period.

Table 12: The wound contraction rates (%) measured after central and peripheral irradiation of LLL (P13, 1min) according to the groups and the elapsed time. (n=20)

group	Time(day) of measurement			
	1st	3rd	5th	7th
Co	100.0±0	59.6±13.3	40.9±11.9	28.5±9.5
PI	100.0±0	62.9±12.1	42.7± 8.8	30.8±9.8

(Co, control group ; PI, peripheral irradiation group)

effect for irradiation method is not siginificant. This means that there was no difference in the effectiveness of the two irradiations as measured by the wound contraction rates.

The means of wound contraction rates measured according to the pulse type, the most proliferative pulse (P13, 14.81 mJ/cm^2) and the least proliferative pulse (P7, 1.06 mJ/cm^2), and the elapsed time using the central irradiation

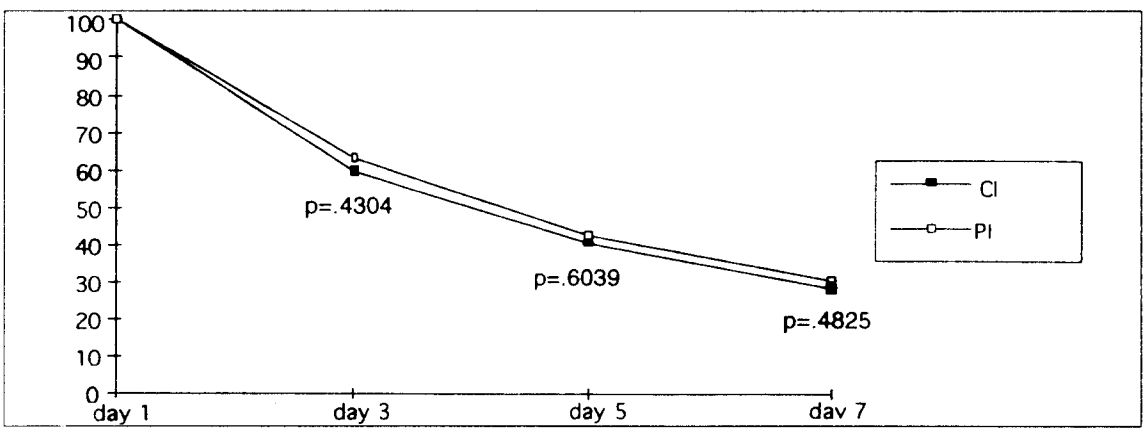

Figure 8: Linear graph showing the changes in the rate of wound contraction size, comparing central irradiation group (CI) with the peripheral irradiation group (PI).

Table 13: Results of ANOVA test for all rates of wound contraction measured after LLLI (P13, 1min) according to the irradiation type and the elapsed time.

	DF	Sun of Square	Mean Square	F-Value	P-Value
Irradiation Type	1	126.12	126.12	0.61	0.437
Subject(Group)	36	7352.72	204.24		
Category for time	3	107918.9	35972.9	674.7	<.0001
Category for time+Irradiation	3	53.47	17.82	0.33	0.800
Category for time+Subject	108	5757.72	53.31		

Table 14: The wound contraction rates (%) measured after central irradiations according to the pulse type and the elapsed time. (n=11)

group	Time(day) of measurement			
	1st	3rd	5th	7th
Co	100.0±0	42.6±19.8	28.2± 15.2	63.8±151.7
PI	100.0±0	46.9±19.5	66.1±131.5	55.1± 88.7

(Co, control group ; PI, peripheral irradiation group)

are given in Table 14. The results of repeated measures ANOVA test according to the groups and the elapsed time are given Table 15 and The change of two groups over time can be seen in Figure 9. The between-group main effect for pulse type is not significant. This means that there was no difference in the effectiveness of the two pulses with central irradiation as measured by the wound contr-

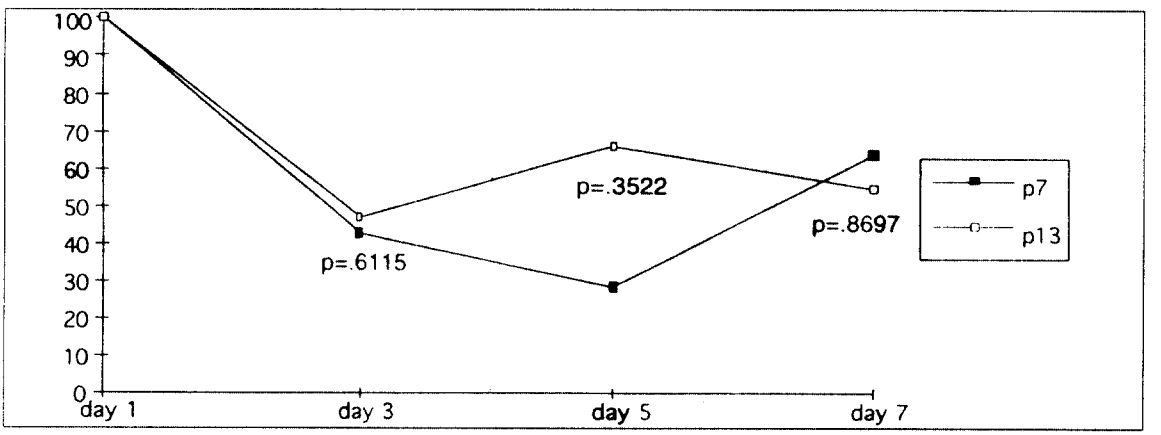

Figure 9: Linear graph showing the changes in the wound contraction rate of P7 and P13 groups, irradiated centrally.

Table 15: Results of ANOVA test for all rates of wound contraction measured after cental irradiation according to the pulse type (P7 and P13) and the elapsed time.

	DF	Sun of Square	Mean Square	F-Value	P-Value
Pulse	1	1544.18	1544.18	0.19	0.664
Subject(Group)	20	159759.44	7987.97		
Category for time	3	43263.73	14421.24	2.60	0.059
Category for time+Puise	3	6923.74	2307.91	0.41	0.741

action rates.

Consequently it can be concluded that although S. aureus was also stimulated by LLLI, the healing of wound infected with S. aureus was accelerated by LLLI regardless of direct or indirect irradiation, therefore, LLLI exerts a significant biostimulative effect on the healing of infected wound.

Ⅳ. Discussion

Kim et al.[12] suggested that *Streptococcus mutans* could be stimulated by LLLI(GaAs) *in vivo*, and similar modulation could potentially occur in other bacteria exposed to LLLI. The results indicated that LLLI increased the growth of *S. mutans* significantly. It was, therefore, postulated that low level laser radiation had same effects for all kinds of cells.[14] *Staphylococcus aureus* is the common cause of skin infections. For this reason, Staphylococcus aureus was used to infect the wounds in this study[12].

Biostimulative effect of LLLI on S. mutans was the most effective when the energy density was at 337.0 mJ/cm^2.[12] The number of *Candida albicans* was markedly increased with LLLI in groups using 2.12 and 6.37 mJ/cm^2 of energy densities.[13] Energy densities tested in this study varied from 1.06 mJ/cm^2 to 88.86 mJ/cm^2 and the results indicated that the most proliferative energy density was 29.62 mJ/cm^2. In previous studies the most effective irradiation interval was not studied, It was, therefore, not possible to compare the energies used in their studies.

Mester et al.[3] reported a clinical study which indicated an accelerating effect of the low output laser on the healing of wound and skin ulcers that were slow in healing. Mashiko et al.[15] also reported the wound- healing effect of the laser in an incised lesion on the back of rats. A series of experiments has been arranged to examine the influence of laser irradiation on the wound healing process .[3,16-19]

There were many studies reported that effects of LLL irradiation under normal condition, however, few study was undertaken by pathologic condition. It was suggested that the effect of LLL irradiation under pathologic condition is still unknown. Lee et al. reported that the closure rate of infected wound was significantly increased and the incidence of swelling, indicating the spread of inflammation was significantly decreased in LLL irradiation group.[14] In their study the frequency of used pulse was 1000 Hz with 2mW of average output power and the energy density was approximately 76.4 mJ/cm^2. The effect of LLLI on the proliferation of *S. aureus* was not included. Therefore, the experiments for *S. aureus* were performed prior to those for the wound healing effect of LLLI to determine the most or the least proliferative pulse, the effective energy density of LLLI and irradiation interval in the present study.

In the experiment for the irradiation time of this study, the proliferation of *S. aureus* was increased the most under 29.62 mJ/cm^2 (2 min) of energy density ($p<.0001$) than 14.81 (1 min) and 44.43 mJ/cm^2 (3 min) of energy densities ($p=.0105, .002$) and rahter decreased under 59.24 mJ/cm^2 (5 min) of energy densities($p=.0003$), as compared with control group. In the experiment for the irradiation interval, to irradiate 29.62 mJ/cm^2 of energy density every 4 hours was more effective to stimulate the bacterial growth than to irradiate higher energy densities with longer interval, although total energies of groups in that experiment were same respectively. These results support a possible mechanism of LLL-living cell interaction as sugge-

sted by Lubart et al.[20].

Singlet oxygen ($^1O_2$) at ^{1}(Dg) is a highly reactive intermediate. It has a sufficiently long half-life in the biological environment (of the order of 1 ms) to react chemically. Singlet oxygen at large amounts rapidly oxidizes a large variety of biological molecules,[21] damages DNA and is responsible for cell destruction. At low concentration, on the other hand, singlet oxygen can modulate biochemical processes taking place in the cell and can trigger immune regulation.[22] Kanofsky et al. suggest that singlet oxygen is a significant biochemical intermediate which may have an important role in biostimulation.[23,24] It is believed that by irradiating damaged tissues with LLL, small amounts of singlet oxygen are produced and are responsible for tissue regeneration.[20]

Lubart et al.[25] reported that LLLI is dependent on wavelength and is limited to a specific energy density. There is a minimum threshold of energy flux below which there is no effect, and a maximum threshold beyond which the effect is reversed. Karu[21] also reported that irradiation of cells with visible light of one and the same wavelength but with different energy doses has a positive effect; acceleration of cell division at low doses, and a negative effect; death of cells at high doses. The explanations of the results are; At low doses of laser irradiation, small amounts of $^1O_2$ are produced which have a positive effect on the cells and at high doses of energy, large amounts of $^1O_2$ are produced and causes a photodynamic damage.[26,27]

It is clear that, in order to interact with tissue, light has to be absorbed by chromophores in the cell. Lubart et al.[20] found that singlet oxygen is generated in the cells during HeNe (630 nm) irradiation. Porphyrins have an intense absorption band in the 360 nm region, and four additional bands with decreasing intensity at 502 nm, 540 nm, 560 nm and 630 nm. Porphyrins are known to be excellent photosensitizers through generation of the lowest excited singlet state of the oxygen. Since singlet oxygen is endowed with a very high reactivity, it usually exerts a damaging action in biological systems. This, of course, explains the cellular destruction after certain doses of laser irradiation.

In this study, however, GaAs semiconductor laser, of which wavelength is 904 nm, was used instead of HeNe laser(630nm). As mentioned above, porphyrins do not have absorption bands in the 904 nm region. Nontheless, GaAs laser irradiation increased the growth of *S. aureus* in a specific range of energy density significantly. Therefore, Singlet oxygen generation theory failed to explain this result, although it can be partially acceptable in the region of visible light, and a different mechanism should be considered.

To explain the biostimulation effect of low level irradiation at 633 nm, Karu[21] proposed a chain of molecular events starting with the absorption of light by a photoreceptor, which leads to signal transduction and amplification, and finally results in the photoresponse. This model also suggests an explanation for why radiation at 904 nm can produce biological effects similar to those produced by radiation at 633 nm. In this model, radiation at 633 nm triggers, probably by photoactivating enzymes in the mitochondria, a cascade of molecular events leading to the photoresponse. Smith[28] suggested that radiation at 904 nm produces the same final response, but initiates the response at the membrane level (probably through photophysical effects on Ca++ channels) at about halfway through the total cascade of molecular events that leads to biostimulation.

It can be summarized that if the biological effect of low level visible light therapy is through photochemistry (probably the photoactivation of enzymes), the biological effect of infrared radiation is through molecular rotations and vibrations. Abergel et al.[29,30] reported that the irradiation of fibroblasts in culture either at 633 nm or at 904 nm increased the synthesis of collagen. Kim et al.[12,13,31] reported that GaAs laser (904 nm) enhanced the protein and DNA synthesis and accelerated the proliferation of the gingival fibroblast. The growth of *S. mutans and C. albicans* coincide with the suggestion that Smith has proposed. During the animal experiment, general anesthesia was required for rats to be irradiated with LLL every session. Thus, the authors used 2-day interval, instead of 4-hour interval because frequent general anesthesia for rats were able to increase the mortality rate during the experiment.

Kim et al.[12] and Lee et al.[14] also suggested a hypothesis on the acceleration of healing in the infected lesion following GaAs LLLI. They postulated that the biostimulation of LLLI in the surrounding normal tissue predominated over the destructive irritation due to the bacterial growth in the infected lesion. In this study the most and least proliferative pulses were investigated to confirm that hypothesis. The most proliferative pulse was P13 (14.81 mJ/cm^2) and the least proliferative pulse was P7 (1.06 mJ/cm^2) among the pulses used in this study. The results from animal study, however, showed that the healing of wounds infected with *S. aureus* was accelerated by LLL irradiation regardless of the pulse type and the irradiation type.

The experiment for the growth of tissue cell of rats was not performed to investigate the effective pulse to stimulate the cell growth after LLLI. Therefore, it can not be told that pulses and energy densities used in this study on the proliferation of S. aureus would have a positive or negative effect on the tissue level as well as the cell level. The results obtained from this study, however, showed that both pulse types of LLLI stimulated the healing of infected wound. It is believed that the reason both pulses were effective was that the media confined in the petridish had some limitations in the proliferation of *S. aureus in vitro*, but the cells of vital tissues were stimulated unlimitedly by LLLI *in vivo*. It is most likely, therefore, that some inhibitory doses of LLLI in the cell culture level might be a stimulatory doses in the tissue or organ level.

On the basis of the results in this study, the healing of infected wound was stimulated in the case of direct irradiation on both the wound and *S. aureus* at a time as well as in the case of indirect irradiation around the infected wound. This means that the biostimulation of LLLI in the surrounding normal tissue predominates over the destructive irritation via an increased bacterial growth on the infected lesion. GaAs laser light has deeper peneration depth than that of HeNe laser light and it is also believed that GaAs laser is more beneficial in stimulating the sound tissue below the wound.

The first-discovered and most widely used mitogen is PHA(phytohematogglutinin), a specific mitogen for T-lymphocytes (70-80% of all population).[32,33] Characteristics of biochemical and morphological reactions in lymphocytes under the action of PHA are fully investigated. In the long-term response to LLLI of lymphocyte, Karu[34] reported that irradiation with He-Ne laser induced some early changes in lymphocytes similar to those caused by PHA (activation of transcription;

Ca^{2}+ influx). At the same time, the irradiation itself did not cause the proliferation of lymphocytes, but elevated the proliferation level induced by PHA.

As a rule, the irradiation increased the effect as compared with the action of PHA alone. Maximal effect of irradiation was found for PHA concentrations 1.0 and 2.0 mg/ml. At very low (0.5 mg/ml) as well as at higher (4.0 mg/ ml) PHA concentrations the effect of irradiation was less pronounced. The irradiation increased the DNA synthesis as compared with the action of PHA alone. The mechanism of this boosting effect of irradiation is unclear, although Karu explained this with part of metabolic processes under the action of light and ATP extrasynthesis of laser radiation. If the boosting effect of LLLI were proved in the future, it would be very interesting for the investigating of the materials which cause the boosting effect in the healing of infected wound.

Theoretically, the potential for rotation and vibration by the action of infrared radiation on the membrane molecules that make up the calcium channels could alter the functionality of these channels. This potential, thereby, provide a mechanism for the therapeutic effect of low level infrared radiation. An important requirement for any theory is that it be testable. The techniques of molecular biology lend themselves very nicely to the testing of this theory in the future.

V. Conclusions

LLLI increased the growth of *S. aureus* under specific condition. This condition is P13 (6,000 Hz, 14 mW average output power, 4 hour interval, 2 min irradiation: 1680 mJ). Nontheless, when LLLI(P13) was irradiated on the wound infected with *S. aureus*, the healing was significantly accelerated.

Comparing the healing effects of central and peripheral irradiations, there were no significant differences between the effects of two irradiations. It can be, therefore, concluded that GaAs LLLI facilitates the healing in the infected wound via an cellular activity. The biostimulation effect of LLLI in the surrounding normal tissue predominates over the destructive irritation due to the bacterial growth in the infected lesion.

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국문초록

저출력레이저조사가 *Staphylococcus aureus* 에 감염된 창상에 미치는 영향

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저수준레이저를 이용하여 창상이나 병소의 치유과정에 대한 효과를 조사하기위하여 많은 연구가 시행되었다. 연구에 의하면 갈륨비소 레이저광이 생체자극효과를 가진다고 하며, 저수준레이저를 조사하면 단백질과 핵산(DNA) 합성을 자극하여 치은섬유아세포의 증식을 촉진한다고 보고하였다. 외상병소나 근육병소의 치료에 사용된 레이저치료법에 관한 관심이 점증함에 따라 저수준레이저요법 (LLLI)의 치유효과를 설명하기위하여 분자생물학적수준의 연구를 시행하기에 이르렀다. 보고에 의하면 *Mutans Streptococcide* 는 LLLI를 사용시 증식이 촉진되며, 다른 세균에서도 유사한 증식효과가 나타날 것이라고 주장하였다. 그러므로 LLLI가 피부감염을 야기하는 가장 흔한 원인인 *Staphylococcus aureus* 도 마찬가지로 증식이 촉진되는 지를 조사해볼 필요가 있으며, 또한 감염과 같이 특정 병적 상태에서의 저수준레이저광의 효과는 아직까지 명확하게 밝혀져 있지지지 않았다. 그러므로 본 연구의 목적은 첫째, *Staphylococcus aureus* 의 증식에 대한 저수준레이저광의 효과를 조사하는 실험이며, 둘째 *Staphylococcus aureus* 로 감염된 피부창상에 대한 저수준레이저광의 효과를 판정하는데 있다. 34개의 *Staphylococcus aureus* 배양표본을 사용하여 48시간의 세포주기동안 조사기간과 조사시간, 그리고 레이저 펄스(laser pulse)형에 따라 3가지 실험을 시행하여 증식에 가장 효과적인 상태와 가장 비효과적인 상태의 갈륨비소 반도체 레이저펄스를 결정하였다. 이후 지름 약 6 mm 의 개방창상을 44마리 백서의 양측 대퇴부에 형성하여 모든 창상에 *S. aureus* 를 감염시켰다. 모든 표본은 펄스형과 조사방법(중앙조사법과 주변조사법)에 따르는 실험을 하기 위하여 4가지로 분류하였다. 각 백서의 양측 창상중 하나는 1, 3, 5, 7일 마다 각 실험의 방법에 따라 레이저를 조사하고 실험동물의 다른 창상은 대조군으로서 사용하였다. 모든 창상의 면적은 실험 1, 3, 5, 7 일째에 일정한 거리에서 사진촬영하여 면적계를 이용, 측정한 후 통계적인 의의를 조사하였다. 본 연구의 결과는 저수준레이저는 특정 조건하에서 *S. aureus*의 증식을 촉진하였다. 그러나 *S aureus* 에 감염된 창상을 저수준레이저로 조사시 치유가 촉진되었다. 중앙조사법과 주변조사법에 의한 창상치유효과는 통계적인 의의가 보이지 않았다. 따라서 결론적으로 *S. aureus*에 감염된 창상에 직접 또는 간접이든 pulse의 종류에 관계없이 조사하는 경우 치유효과가 나타나는 것은 정상주위 조직의 LLLI 자극효과가 염증의 확산을 억제한다고 말할수 있다.