EFFECTS OF LOW INCIDENT ENERGY LEVELS OF INFRARED LASER IRRADIATION ON THE PROLIFERATION OF *CANDIDA ALBICANS*. PART III: A STUDY ON THE INTERIRRADIATION INTERVAL

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This study was performed to investigate the biostimulation effects of low level laser therapy (LLLT) on the fungus, *Candida albicans*, according to the interval of irradiation during the cell cycle. Samples were randomly divided into 5 groups : pulse 7 with 2 hour interval (P7-2Hr), pulse 7 with 4 hour interval (p7-4Hr), pulse 15 with 2 hour interval (P15-2Hr), pulse 15 with 4 hour interval (P15-4Hr) and control (Co). Each of the experimental samples was irradiated for 1 minute with a 2 hour interval, or for 2 minutes with a 4 hour interval, during 28 hours of the cell cycle of *C. albicans*, and the optical density of the cultures as a marker of cell growth was assessed by spectrophotometry every 2 hours. It was found that there was a significant difference in cell growth between the 2 hour and 4 hour interval laser irradiation groups in favour of the 4 hour groups, although the total energy of both groups using an identical pulse setting was equal during the study. There was also a significant difference between the 4 hour interirradiation groups and the control group, but no significant difference between the 2 hour interirradiation groups and the control group. It is, therefore, concluded that frequent irradiation sessions using a short interirradiation interval with LLLT on cells do not induce any significant biostimulation effect on the cell growth at optimal dose or even at low doses of LLLT, and that an appropriate irradiation interval is required for the acceleration of cell growth.

Key words: LLLT, *Candida albicans*, cell growth, biostimulation, irradiation interval

Introduction

It has been suggested that low incident energy levels of laser irradiation have a favourable influence on treatment of gingival inflammation through inducing a change of rate in the composition of oral flora.⁽¹⁾ Kim et al.^(2,3) previously reported that infrared gallium-arsenide (GaAs) laser irradiation at low level laser therapy (LLLT) levels stimulated the growth of *Streptococcus*

mutans but the growth rate of *S. mutans* was not always in accordance with the frequency or fluence of the LLLT laser. Lubart et al.⁽⁴⁾ suggested that LLLT is dependent on wavelength, is not thermal and is limited to a specific energy density. Kim et al. also suggested, in their previous study on LLLT effects on *C. albicans*, that specific laser pulses are recommended to produce biostimulative effects on the specific tissue or cells, although the biostimulation effect was seen to be dose dependent.⁽⁵⁾ In a recent study, however, it was found that there was no difference between the control group

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Fig 1: Schematic illustration of the relationship between the laser probe and the culture flask. The distance between the distal end of the probe and the base of the flask was set using an IR beam viewer to ensure that the laser beam involved the entire base of the flask.

and any other group irradiated with a short inter-irradiation interval of 2 hours.⁽⁶⁾ Therefore, it is suggested that frequent irradiation of LLLT 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 acceleration of cell growth. The present study was therefore performed to investigate the effect of LLLT on the growth of *Candida albicans* under different irradiation intervals during the cell cycle, using an infrared GaAs laser.

Materials and Methods

Laser Apparatus.

The laser used in this work was the BIOLASER (Dong Yang Medical, Korea) using a GaAs semiconductor as the medium. It is a pulsed infrared laser apparatus with a wavelength of 904 nm and a peak output power of 27 W. Pulsed rates used in this study were pulse 7 (p7: 500 Hz, 1 mW average output power) and pulse 15 (P15: 10 kHz, 27 mW average output power).

Micro-organism.

Candida albicans (*C. albicans*, American Type Culture Collection No 28366) was used in this study.⁽⁷⁾

Spectrophotometer

To evaluate the possible growth of *C. albicans in vitro*, a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, NY, USA) was used to measure the optical density of cell culture broth. The spectrophotometer was set at 600 nm. The reference liquid was identical brain heart broth media but without cells.

Flask used to measure OD

Specially designed flasks were made to measure OD repeatedly. A Pyrex test tube (20 ml) was attached to the neck of an Erlenmeyer flask (300 ml) at an angle of about 20 degrees (Figure 1).

Culture

C. albicans was chosen for this study, because this microorganisms is commonly seen as normal flora in the oral cavity, and it was maintained on a Sabouraud dextrose agar plate (Difco, USA) at 4°C.

Media (SDA) was prepared with 20 g glucose, 10 g Neo-peptone, 15g Agar in one litre of distilled water (pH 7.0). A single colony of *C. albicans* 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 30°C (Water Bath Incubator BT-46, Yamato, Japan). The seed culture was

Fig 2: Line graph showing the changes in the growth of *Candida albicans* (as measured photospectrometrically using the optical density of medium as a cell growth marker) for each of the four LLLT-irradiated groups and the unirradiated control group

inoculated into .100 ml of medium. Temperature was controlled to 30°C and culture pH was maintained at 7.0.

Laser irradiation

The samples were randomly divided into 5 groups according to the pulse type and the irradiation interval: pulse 7 with 2 hour interval (P7-2Hr), pulse 7 with 4 hour interval (P7-4Hr), pulse 15 with 2 hour interval (P15-2Hr), pulse 15 with 4 hour interval (P15-4Hr) and the nonirradiated control (Co) groups.

The author used a laser beam detector to ascertain the optimum distance from the laser probe to the base of flask, because the total area of flask base must be covered completely by the laser beam from the probe.

Using a clamp laser probe was precisely positioned vertically about 13 cm above the middle of media in the bottom of the flask (Figure 1).

After the inoculation of the seed culture, some samples of P7-2Hr and P15-2Hr groups were irradiated with the laser of pulse 7 and 15 for 1 minute in the beginning of experiment and every 2 hours during 28 hours of cell growth, and the other samples of P7-4Hr and P15-4Hr groups were irradiated with the corresponding pulsed laser for 2 minutes in the beginning of experiment and every 4 hours during the cell growth.

All samples were cultivated in a shaking incubator (100 rpm) at 30°C. The optical density of all sample was measured with spectrophotometer every 2 hours for

Table 1: Mean and standard deviations of the optical densities measured according to the pulse type and the interval during the growth cycle of *Candida albicans*.

Cell cycle (hrs)	Group				
	Control	P7-2Hr	P7-4Hr	P15-2Hr	P15-4Hr
14	0.010 ± 0.000	0.010 ± 0.000	0.025 ± 0.007	0.015 ± 0.007	0.045 ± 0.007
16	0.030 ± 0.000	0.040 ± 0.000	0.080 ± 0.014	0.065 ± 0.007	0.115 ± 0.007
18	0.090 ± 0.014	0.075 ± 0.007	0.220 ± 0.028	0.140 ± 0.057	0.280 ± 0.000
20	0.275 ± 0.035	0.255 ± 0.007	0.500 ± 0.000	0.275 ± 0.035	0.540 ± 0.014
22	0.540 ± 0.85	0.505 ± 0.021	0.670 ± 0.014	0.540 ± 0.057	0.725 ± 0.035
24	0.790 ± 0.085	0.775 ± 0.35	0.900 ± 0.000	0.795 ± 0.064	0.965 ± 0.035
26	0.960 ± 0.000	0.935 ± 0.021	1.065 ± 0.021	0.960 ± 0.057	1.050 ± 0.071
28	1.025 ± 0.035	1.000 ± 0.000	1.125 ± 0.035	1.075 ± 0.035	1.125 ± 0.035

the 28 hours of the *C. albicans* cell cycle. Energy densities for the P7-2Hr, P15-2Hr, P7-4Hr and P15-4Hr groups for each session were 1.06 mJ/cm², 28.56 mJ/cm², 2.12 mJ/cm² and 57.12 mJ/cm², respectively. Total energy fluences of the experimental groups during this study were set to be equal under an identical pulsed laser: 14.84 mJ/cm² for 14 sessions in P7-2Hr group and for 7 sessions in P7-4Hr group, and 399.84 mJ/cm² for 14 sessions in P15-2Hr group and for 7 session in P15-4Hr group.

Statistical analysis

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

Results

The means of all groups classified according to the pulse type with different intervals and time are given in Table 1, and the changes in the OD, and hence the cell growth or otherwise, are shown in Figure 2 for all groups. To test whether there were any differences in the pattern of change over time between the two pulse types and intervals, Repeated measures ANOVA was used in this study and the result is seen in Table 2. This result means that averaged over the elapsed times there were significant differences in the means of all groups as measured by the optical densities ($p < 0.001$), and there was significant pulse-by-time interaction indicating that the patterns of changes in means of optical densities over time differ depending on the pulses with different intervals ($p = < 0.0024$). Using the multiple comparison test, the significance could be seen among most groups (Table 3), however there were no significant differences between the means of the control group and any of the 2 hour interval groups. This result means that there is no biostimulation effect of LLLT on the *C. albicans* in the case of frequent irradiation with a 2 hour interirradiation interval.

Discussion

This study was performed to investigate the effect of LLLT on the growth of *C. albicans* under different interirradiation intervals. Pulse 7 and 15 settings in the laser system we were using were chosen in this study because pulse 7 has the lowest average output power of 1 mW and the pulse 15 had the highest average output power of 27 mW among the range of pulse settings available from the laser apparatus we used in this study. The authors' previous study⁽⁷⁾ did not demonstrate the effect of LLLT according to the pulse type when the interirradiation interval was less than two hours, which suggested that frequent irradiation with a short interirradiation interval has a rather inhibitory effect on the cell culture, instead of a stimulatory one. The authors, therefore, used two interirradiation intervals of 2 hours and 4 hours in this study. The authors irradiated the laser beam for 1 minute and 2 minutes per treatment for the 2 hr and 4 hr groups, respectively, to equalize the total energy delivered to both groups since it was believed that the effect of irradiation interval on the cell growth could be detected under an identical total energy.

The total incident energies of both the 2 hour interval group and the 4 hour interval group are identical, but the results are obviously different (Figure 2). There was no significant difference in cell growth between control and 2 hour interval groups (P7-2Hr and P15-2Hr), as described in the previous study.⁽⁶⁾ Significant differences, however, could be seen between the control and one of the 4 hour interval groups (P7-4Hr and P15-4Hr) and between the 4 hour interval group and the 2 hour interval group (Table 3). These results show that a 4 hour interirradiation interval of LLLT at the parameters and with the protocol used in the present study is more effective than a 2 hour interirradiation interval to accelerate *C. albicans* cell growth in vitro regardless of the pulse type and energy density, although there was a significant difference between P7-4Hr and P15-4Hr groups, indicating a dose dependency effect for these groups.

Table 2: Results of Repeated measures ANOVA test for the optical densities of all groups measured according to the pulse type with different interirradiation interval and exposure time

Item	DF	Sum of Squares	Mean Square	F-Value	P-Value
Groups	4	.311	.078	126.268	.000
Time	7	12.452	1.779	1398.310	.000
Time*Group	28	.0098	.003	2.763	.002
Residual	35	.045	.001	—	—

Table 3: Results of multiple comparison test (Fisher's PLSD) for all groups

Test groups	Mean Difference	Critical Difference	P-Value	Significance
Co : P7-2Hr	0.016	0.023	0.1348	—
Co : P7-4Hr	-0.108	0.023	< 0.0001	s
Co : P15-2Hr	-0.018	0.023	0.0936	—
Co : P15-4Hr	-0.141	0.023	< 0.0001	s
P7-2Hr : P7-4Hr	-0.124	0.023	< 0.0001	s
P7-2Hr : P15-2Hr	-0.034	0.023	0.0120	s
P7-2Hr : P15-4Hr	-0.156	0.023	< 0.0001	s
P7-4Hr : P15-2Hr	0.090	0.023	0.0002	s
P7-4Hr : P15-4Hr	-0.033	0.023	0.0139	s
P15-2Hr : P15-4Hr	-0.123	0.023	< 0.0001	s

s: significant (95%)

The energy densities of the P7-2Hr, P15-2Hr, P7-4Hr and P15-4Hr used each session in this study were 1.06 mJ/cm², 28.56 mJ/cm², 2.12 mJ/cm² and 57.12 mJ/cm², respectively, but the total energy fluences used in all sessions were 14.84 mJ/cm² in the 2 hour interval groups and 399.84 mJ/cm² in the 4 hour interval groups. Comparing these doses of LLLT to those LLLT doses used in the previously reported studies,^(5,8,9) the dose delivered to each of the groups irradiated for a session in this study was similar to the doses used in the previous study, but the total energy fluence of each pulsed laser irradiated in this study was not easy to compare with those of previous studies. It is thought, however, that the total energy fluences in this study were not larger than most of those in the previous studies.^(3,9,10)

It is thought that the mechanisms generated or activated by frequent laser irradiation with a shorter interirradiation interval had no stimulation effect on the cell growth, and could possibly be explained as follows: First, singlet oxygen at low concentration can modulate biochemical processes taking place in the cell.⁽¹⁰⁾ It has been therefore suggested that singlet oxygen is a significant biochemical intermediate which may have an important role in biostimulation. In large amounts, on the other hands, singlet oxygen rapidly oxidizes a large variety of biological molecules,⁽¹²⁾ damages DNA and is responsible for cell destruction. Lubart *et al.*⁽¹³⁾ suggested that singlet oxygen can be photoproduced by the natural porphyrins in the cell. Porphyrins have an intense absorption in the visible shorter wavelength blue region around 440 nm and four additional absorption

bands with decreasing intensity between 500 and 630 nm. In this study, however, the wavelength of laser used was 904 nm, which is not in the visible waveband. To establish the above hypothesis for the effect of frequent irradiation, therefore, some studies should be planned to elicit or clarify the biostimulation effect of LLLT at 904 nm wavelength on porphyrins.

Second, laser irradiation is also assumed to intensify the formation of a transmembrane electrochemical proton gradient in mitochondria.⁽¹⁴⁾ Thus, at low doses, the additional Ca⁺⁺ transported into the cytoplasm triggers mitosis and enhances cell proliferation. At higher laser doses too much Ca⁺⁺ is released, which completely exhausts the cell energy and the intracellular osmotic pressure causes the cell to explode. Based on these results and previous hypothesis, it is conceivable that frequent LLLT irradiation with a short interirradiation interval at least on the cells in an *in vitro* setting does not cause any biostimulation effect on the cell at a predetermined optimal dose per exposure, or even at repeated low doses. It is, therefore, important to recognize that an appropriate irradiation interval should be obtained in order to achieve acceleration of cell growth.

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