EFFECTS OF LOW INCIDENT ENERGY LEVELS OF INFRARED LASER IRRADIATION ON THE PROLIFERATION OF *CANDIDA ALBICANS*

PART III : A STUDY ON THE INTERVAL OF IRRADIATION

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CONTENTS

I. Introduction

It was suggested that the LLL irradiation had a favourable influence on the gingival inflammation with the change of rate in the composition of oral flora.[1] Kim *et al.*[2,3] reported that infrared gallium-arsenide(GaAs) laser irradiation at LLLT levels stimulated the increase of *Streptococcus mutans* growth but the growth rate of *Streptococcus mutans* is not always in accordance with the frequency or fluence of 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 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.[5] In recent study, however, it was found that there were no difference between the control group and any group of others irradiated with 2 hours of short interval.[5]. Therefore, it is assumed that frequent irradiation of LLLT on the cells has no biostimulation effect or rather inhibitory effect on the cell growth and proper interval of irradiation should be investigated for the acceleration of cell growth.

This study was performed to investigate the effect of LLLT on the growth of *Candida albicans* under different irradiation intervals during the cell cycle, using infrared GaAs laser.

II. Materials and Methods

Materials

1. Micro-organism

The micro-organism used in this study was *Cadida albicans*, because it is common seen as normal flora in oral cavity. *Cadida albicans*, ATCC #28366 was obtained from American Type Culture Collection. The strain was maintained by transferring to fresh stock culture media(Sabouraud dextrose agar) each month and storing at 4℃.

2. Culture Media

Media (SDA) was prepared with 20g glucose, 10g Neo-peptone, 15g Agar in liter of distilled water(pH 7.0). A single colony of *Cadida albicans* developed on the plate of the stock

culture medium was transferred to 20ml of the seed culture medium and incubated in a rotary shaking incubator at 30℃(Water Bath Incubator BT-46, Yamato, Japan). The seed culture was inoculated into 100ml of the medium. Temperature was controlled to 30℃ and culture pH was maintained to 7.0.

3. Laser Apparatus.

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

4. Flask used to measure OD

Special flasks were made to measure OD repeatedly. A pyrex test tube(20ml) is attached in the neck of erlenmeyer flask(300ml) with about 25 degree of slope (Figure 1).

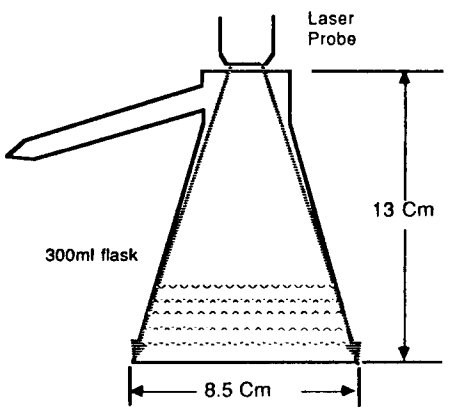

Figure 1. Schematic illustration of the relationship between the laser probe and the tissue culture dish.

5. Spectrophotometer

To evaluate the growth of *Cadida albicans*, spectrophotometer (Sepectronic 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.

Methods

The samples were randomly divided in 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 shame-irradiated control (Co) groups.

The author used the holder to fix the laser probe vertically about 13cm apart from the middle position between top and bottom of media in the flask(Figure 2). The author used the laser beam detector to examined the distance, from the laser probe to the base of flask, which the total area of flask base could be covered completely by the laser beam from the probe.

After the inoculation of seed culture, samples of P7-2Hr and P15-2Hr groups were irradiated with the laser of pulse 7 and 9 for 1 munute in the begining of experiment and every 2 hours during 28 hours of cell cycle, and samples of P7-4Hr and P15-4Hr groups were irradiated with the corresponding pulsed laser for 2 minutes in the begining of experiment and every 4 hours during the cell cycle.

All samples were cultivated in a shaking incubator (100rpm) at 30℃. The optical density of all sample was measured with spectrophotometer every 2 hours for 28 hours of cell cycle. Energy densities of samples for each session were 2.12, 57.32, 4.24 and 114.64 mJ/cm^2 in P7-2Hr, P15-2Hr, P7-4Hr and P15-4Hr groups respectively. Total energy fluences of experimental groups during this study were set to be equal under identical pulsed laser:29.68mJ/cm^2 for 14 sessions in P7-2Hr group and for 7 sessions in P7-4Hr group, and 802.48 mJ/cm^2 for 14 sessions in P15-2Hr group and for 7 sessions 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.

III. Results

The means of all groups classified according to the pulse type with different interval and the elapsed time are given in Table 1, and the changes of OD in all groups are shown in Figure 2. To test whether the pattern of change over time is the same for the different pulse types and intervals, Repeated measures ANOVA was used in this study and the result was 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 ($p<.0001$), and there was significant pulse-by-time interaction indecating that the patterns of changes in means of optical densities over time differ depending on the pulses with different intervals ($p=.0024$). Using the multiple comparison test, the significances could be seen among most of groups(Table 3), however there was no significant difference between the means of control group and anyone of 2 hours interval groups. This study suggested, consequently, that there is no biostimulation effect of LLLT on the *Cadida albicans* in the case of frequent irradiation with 2 hour interval.

Table 1. Mean and standard deviations of the optical densities measured according to the pulse type and the irradiation interval during the cell cycle of *Cadida albicans*

Group	14 Hr	16 Hr	18 Hr	20 Hr
Co	.010±.000	.030±.000	.090±.014	.275±.035
P7-2 Hr	.010±.000	.040±.000	.075±.007	.255±.007
P7-4 Hr	.025±.007	.080±.014	.220±.028	.500±.000
P15-2 Hr	.015±.007	.065±.007	.140±.057	.275±.035
P15-4 Hr	.045±.007	.115±.007	.280±.000	.540±.014
Group	**22 Hr**	**24 Hr**	**26 Hr**	**28 Hr**
Co	.540±.085	.790±.085	.960±.000	1.025±.035
P7-2 Hr	.505±.021	.775±.035	.935±.021	1.000±.000
P7-4 Hr	.670±.014	.900±.000	1.065±.021	1.125±.035
P15-2 Hr	.540±.057	.795±.064	.960±.057	1.075±.035
P15-4 Hr	.725±.035	.965±.035	1.050±.071	1.125±.035

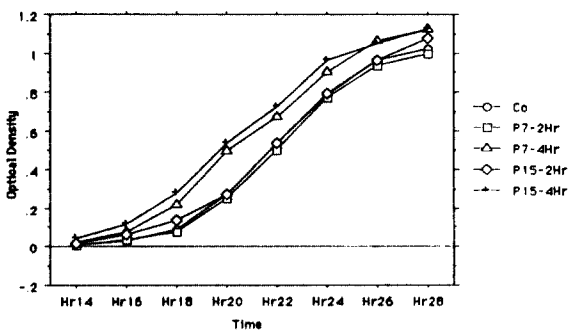

Figure 2. Linear graph showing the changes in the growth of *Candida albicans*(as measured photospectrometrically by optical density of medium) as a factor of each pulse type and postirradiation period, compared with the control medium

Table 2. Results of Repeated measured ANOVA test for optical densities of all groups measured according to the pusle type with different interval and time

Item	DF	Sum of Squares	Mean Square	F-Value	P-Value
P-I	4	.311	.078	126.268	<.0001
Subject(Group)	5	.003	.0006		
Category for Time	7	12.452	1.779	1398.310	<.0001
Category for Time * P-I	28	.098	.003	2.763	.0024
Category for Time * Subjec...	35	.045	.001		

P-I: Pulses with differnet interval

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

	Mean Diff.	Crit. Diff	P-Value	
Co, P7-2Hr	.016	.023	.1348	
Co, P7-4Hr	-.108	.023	<.0001	S
Co, P15-2Hr	-.018	.023	.0936	
Co, P15-4Hr	-.141	.023	<.0001	S
P7-2Hr, P7-4Hr	-.124	.023	<.0001	S
P7-2Hr, P15-2Hr	-.034	.023	.0120	S
P7-2Hr, P15-4Hr	-.156	.023	<.0001	S
P7-4Hr, P15-2Hr	-.090	.023	.0002	S
P7-4Hr, P15-4Hr	-.033	.023	.0139	S
P15-2Hr, P15-4Hr	-.123	.023	<.0001	S

Ⅳ. Discussion

This study was performed to investigate the effect of LLLT on the growth of *Candida albicans* under different irradiation intervals. Pulse 7 and 15 were used in this study because pulse 7 has the lowest energy density (2.12 mJ/cm^2) and pulse 15 has the highest energy density among pulses produced by laser apparatus used in this study.

Previous study[6] did not demonstrate the effect of LLLT according to the pulse type under 2 hours of interval and suggested that frequent irradiation with short interval has rather inhibitory effects on the cell culture. The authors, therefor, used two irradiation intervals, 2 hours and 4 hours in this study. The authors irradiated the laser beam for 2 minutes and 1 minute respectively to make total energies of them equalized since it was believed that the effect of irradiation interval on the cell growth could be detected under the identical total energy.

Total energies of both 2 hour interval group and 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 control and 4 hour interval groups(P7-4Hr and P15-4Hr) (Table 3). These

results show that 4 hour interval irradiation is more effective than 2 hour interval to accelerate the cell growth regardless of pulse type and energy density, although there was significant difference between P7-4Hr and P15-4Hr groups, indicating dose dependency.[3]

Energy densities of P7-2Hr, P15-2Hr, P7-4Hr and P15-4Hr used each session in this study were 2.12, 57.32, 4.24 and 114.64 mJ/cm^2 respectively, but total energy fluences used in all sessions were 29.68 mJ/cm^2 in 2 hour interval groups and 802.48mJ/cm^2 in 4 hour interval groups. Comparing these doses of LLLT to those of LLLT used in the previous studies[5, 7, 8], the dose of each group irradiated for a session in this study was similar to the dose 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 total energy fluence in this study was not larger than most of them in the previous studies.[3, 9, 10]

It is thought that the mechanisms frequent laser irradiations had no stimulation effect on the cell growth can be explained as follow: First, singlet oxygen at low concentration can modulate biochemical processes taking place in the cell.[11] It was, therefore, suggested that singlet oxygen is a significant biochemical intermediated which may have an important role in biostimulation. At large amounts, on the other hands, singlet oxyen 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. Porphyrin has an intense absorption in the violet region around 440nm and four additional absorption bands with decreasing intensity between 500 and 630nm. In this study, however, wavelength of laser used was 904nm, which was not in the violet region. To establish above hypothesis for the effect of frequent irradiation, therefore, the biostimu-lation effect of LLLT with 904nm of wavelength on the porphyrin should be preceded.

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^{2+} transported into the cytoplasm triggers mitosis and enhances cell proliferation. At higher laser doses too much Ca^{2+} is released, which completely exhausts the cell energy and the intracellular osmotic pressure explodes the cell. Based on these results and previous hypothesis, it is conceivable that frequent irradiation with short interval has rather inhibitory effects on the cell culture, although LLLT is irradiated at low doses.

It is suggested that frequent irradiations of LLLT on the cells do not cause the biostimulation effect on the cell even under the optimal doses of LLLT and proper irradiation interval is required for the acceleration of cell growth.

V. Summary and Conclusion

This study was performed to investigate the biostmulation effects of low level laser therapy (LLLT) on the fungus, *Candida albicans*, according to the interval of irradiation during the cell cycle. Samples were divided into 5 groups which were P7-2Hr, P7-4Hr, P15-2Hr,P15-4Hr and Co. Sample was irradiated for 1 minute with 2 hours or 2 minutes with 4 hours of elapsed time during 28 hours of the cell cycle of *Candida albicans*, and the optical density was assessed by spectrophotometry every 2 hours. It was found that there was significant difference, in the cell growth, between 2 hour and 4 hour interval laser irradiation, although the total energy of each group using identical pulse during exam was equal.

As a result of this study and previous researches, It is suggested that frequent irradia-

tion of LLLT on the cells has no biostimulation effect on the cell growth and proper irradiation interval is required for the acceleration of cell growth.

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Abstract

This study was performed to investigate the biostimulation effects of low level laser therapy (LLLT) on the fungus, *Candida alvicans*, according to the interval of irradiation during the cell cycle. Samples were divided into 5 groups which were P7-2Hr, P7-4Hr,P15-2Hr,P15-4Hr and Co. Sample was irradiated for 1 minute with 2 hours or 2 minutes with 4 hours of elapsed time during 28 hours of the cell cycle of *Candida albicans*, and the optical density was assessed by spectrophotometry every 2 hours. It was found that there was significant difference, in the cell growth, between 2 hour and 4 hour interval laser irradiation, although the total energy of each group using identical pulse during exam was equal.