The Effect of single Low-Power CO_2 Laser irradiation on human PDL fibroblast Proliferation & Differentiation

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The Effect of single Low-Power CO_2 Laser irradiation on human PDL fibroblast Proliferation & Differentiation

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감사의 글

이 작은 결실을 맺을 수 있도록 부족한 저를 항상 따뜻한 관심과 지도로 격려해 주시고 이끌어 주신 김종관 교수님께 깊은 감사를 드립니다. 그리고, 많은 조언과 격려를 해주신 채중규 교수님, 조규성 교수님, 최성호 교수님, 김창성 교수님께 진심으로 감사드립니다.

본 연구 내내 많은 도움을 아끼지 않은 정의원 선생님, 김태균 선생님과 논문 교정을 도와준 상권이형, 유정에게 감사드리고, 치주과 의국원, 특히 3년을 동고동락한 사랑하는 동기들, 익현이형, 경준이형, 지은, 지현, 대석에게도 고마움을 전합니다.

마지막으로, 항상 곁에서 든든하게 후원해주시고, 언제나 끝이 없는 사랑으로 저를 감싸주시는 아버지, 어머니, 장인, 장모님, 누님, 매형, 형님, 처형, 처제에게 감사드리고, 세상에서 가장 사랑하는 아내와 내년 5월에 태어날 아이에게 고마움을 전합니다.

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Abstract

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Low-power laser therapy using CO_2 lasers is known to activate surrounding cells and tissue. Shiozaki et al.(2005) achieved that low-power CO_2 lasers induced not only mineralization but also osteoblast differentiation. And Tajima et al (2003). reported that low-power CO_2 laser accelerates new bone formation within the marrow cavity subjacent to the laser treatment site. In spite of successful clinical and experimental results, the mechanisms underlying low-power CO_2 laser effects are still poorly understood.

The aim of this study is to evaluate the effect of low-power CO_2 laser in growth and proliferation of cultured human PDL fibroblast in vitro and to understand better about the effect of laser on periodontal regeneration. The study also tried to find out the most effective degree of energy and power density where cell proliferation and differentiation to osteoblastic cells highly takes place after laser irradiation.

The experimental groups were divided into 4 groups by applying different irradiating distances from cultured cell to laser tip; 2Cm (Focal spot=∅0.74mm), 3Cm (Focal spot=∅1.04mm) and different irradiating time; 1 second, 3 seconds. And they were applied to laser at a power of 0.5W

with 50Hz under continuous mode. The control group was not applied to laser. MTT and ALP activity test were performed to observe the growth of PDL cells and cell differentiation to osteoblastic cells. The result are as follows.

1. On the 5th day after laser irradiation, statistically significant increase of cells were found in all groups. But, no significant differences were found among groups.

2. Statistically significant increase of ALP activity was seen on the 3rd, 5th, 7th, and 10th day after laser irradiation compared to the baseline. Among this, there were highest ALP activity on 3rd day except 2cm,1sec group. 2cm,1sec group showed increase of ALP activity not statistically significant from the 3rd day to 10th day.

On the 7th and 10th day 3cm,1sec, 3cm,3sec and 2cm,1sec group showed significant high score of ALP activity compared to 2cm,3sec and control group.

In this study, there was a weak effect of low-power CO_2 laser on cell proliferation but there was a better effect on cell differentiation. 2cm,1sec irradiation was found to be the most effective condition for PDL cell differentiation in this study and 3cm, 3sec was found to be efficient.

Key words : CO_2 laser, PDL fibroblast, cell proliferation, cell differentiation, power density, Energy

The Effect of single Low-Power CO_2 Laser irradiation on human PDL fibroblast Proliferation & Differentiation

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I. Introduction

Laser is widely used in oral surgery[12,27], endodontics, periodontology[14] and restorative dentistry[25]. Lasers can be classified as surgical (high power) and non-surgical (low power) according to therapeutic purposes. Particularly low-power laser, has been increasingly used for the treatment of injuries of soft and hard tissue.

Low-power lasers are widely used as tissue stimulator, to improve wound repair and anti-inflammatory and analgesic effects.[5,23]

Experimental reports have also suggested that laser would accelerate wound repair.[2,8,18,23,38,39] This effect could be explained by change in mitotic activity.[24,35] Favourable results were achieved in examinations of hard tissue, as bone fractures in mice showed a faster formation of

bone tissue with a tighter mesh of trabeculae after three weeks of daily irradiation with Helium-Neon laser.[32] And Ozawa et al.(1995) achieved a significant increase in the total area of bone nodules with a Gallium-Aluminium-Arsenide laser(GaAlAs) in a dose-dependent manner.[21] (10.8-108J/cm^2/day) Another studies report that low-power laser promotes growth and differentiation of cells through many experiments.[1,7,13,22-24,26,34] This shows that low-power laser may give positive effects in regeneration of periodontal tissue by promoting growth and differentiation of cells related to regeneration, especially hard tissue regeneration, and this could be applied in destroyed periodontal tissue.[6,19,30]

The energy of wavelength of commercially available CO_2 laser is 10.6 ㎛, which falls in the far infrared range. This particular wavelength of energy is effectively absorbed by water. Consequently, the CO_2 laser would appear to be the best choice for use on hydrated, composite mineralized tissue such as bone.[11] Also, low-power laser therapy using CO_2 lasers is known to activate surrounding cells and tissue. Shiozaki et al.(2005) achieved that low-power CO_2 lasers induced not only mineralization but also osteoblast differentiation.[29] And Tajima et al.(2003) reported that low-power CO_2 laser accelerates new bone formation within the marrow cavity subjacent to the laser treatment site.[31] In spite of successful clinical and experimental results, the mechanisms underlying low- power CO_2 laser effects are still

poorly understood.

The aim of this study is to evaluate the effect of low-power CO_2 laser in proliferation of cultured human PDL fibroblast and to further understand laser effect in periodontal regeneration. In addition, this study tried to find out the most effective degree of energy and power density in which cell proliferates and differentiates to osteoblastic cells after laser irradiation. Through this, we intend to make the base of clinical periodontal regeneration procedure by low-power CO_2 laser therapy.

II. Materials and Methods

PDL cell separation and culture

The human PDL fibroblast was gathered and cultured from a healthy premolar extracted for orthodontic treatment. Before extraction, plaque and calculus were removed with the use of a periodontal curette. The extracted tooth was then rinsed with HBSS to remove the blood and other foreign bodies, the PDL cells were collected from the middle portion of root surface.

α-MEM including 10% fetal bovine serum, 100U/ml penicillin, 100mg/ml streptomycin and 0.5mg/ml amphotericin-B was used as the culture media with an environment of 37℃, 100% humidity and 5% CO_2. Culture medium was changed every 2 or 3 days.

Experiment design

The experiments were divided into 1) MTT assay, 2) ALP detection assay and 3) ALP activity assay. The control group was not applied to laser. Experimental groups were divided into 4 groups by applying different irradiating distances from cultured cell to laser tip; 2Cm(Focal spot=∅0.74mm), 3Cm(Focal spot=∅1.04mm) and different irradiating time; 1 second, 3 seconds.

Application of Laser

Laser-treated specimens were irradiated with a CO_2 laser‡ using a focused beam of 0.74, 1.04mm diameter focal spot, wavelength of 10.6 ㎛. Laser parameters were 0.5W of power delivered at 50Hz with continuous mode. The operator wore the protection glasses for the risk of damaging the eyes due to the direct vision of the laser and the refraction of the beam.

MTT assay

After seeding PDL fibroblast at 96 well plate, the cells were cultured it in α-MEM including 5% FBS and was made sure that cells were attached to the plate. One day after seeding, laser was irradiated and MTT assay was done every 0,3, and 5 days later. Different laser treatment conditions were applied with different distances from cultured cell to laser tip and irradiating time. The distances were 2cm and 3cm with time condition of 1 second, 3 second. And the control group was not exposed to laser.

The samples were measured by using cell proliferation assay kit(CHEMICON International Inc, Temecula, CA, USA) on absorbant of 570nm wave, and prior to measuring, the cultured cells were incubated by removing reagent out of the kit as maker allowed.

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‡ OPELASER O3SII, Yoshida Dental MFG. CO., Japan

ALP detection assay

After seeding PDL fibroblast at 24 well plate, the cells were cultured it in α-MEM including 10% FBS till it become semi-confluenced. Laser was applied and ALP was detected after 0,3, and 15 days. At this time different laser treatment condition was used. Different laser treatment conditions were applied with different distances from cultured cell to laser tip and irradiating time. The distances were 2cm and 3cm with time condition of 1 second, 3 second. And the control group was not exposed to laser.

Detection was done by using TRACP & ALP double-stain kit (Takara Bio Inc, Seta 3-4-1, Otsu, Shiga 520-2193, japan)and samples were viewed under light microscope. Before sample detection, the cells were incubated by removing the reagent out of the kit as the manufacturer allowed. And the control group was not exposed to laser.

The samples obtained in accordance to time were recorded by photographs.

ALP activity assay

After seeding PDL fibroblast at 6 well plate, the cells were cultured in α-MEM including 10% FBS till it become semi-confluenced. Laser was applied and ALP was detected after 0,3,5,7, and 10 days. At this time different laser treatment condition was used. Apply laser at distance, from cultured cell to laser tip, of 2cm, 3cm and apply each

different irradiation time, 1sec, 3sec. And the control group was not exposed to laser.

ALP activity of the cell was measured by the method of Lowry et al.[16] with p-nitrophenyl phosphate as a substrate and was normalized by the total protein content of the cell which was determined by Bio-Rad Protein assay kit. (BioRad 2000, Alfred Nobel Drive Hercules, CA 94547, USA) Briefly, after finishing the laser irradiation, the medium was removed and the cells were washed twice with Tris-buffered saline. The cells were detached from the culture dish with a scraper after the addition of Tris-buffered saline. ALP activity was then assayed using Alkaline Phosphatate Substrate Kit (Wako Pure Chemical Industries, Osaka, japan).

Statistical Analysis

1-way ANOVA and tukey's test for multiple comparison was used to determine the statistical significance of MTT assays and ALP activity between different treatment groups at baseline and at indicated time points after laser irradiation. Repeated Measures ANOVA was used to determine the statistical significance of the MTT and ALP activity within groups in comparison with baseline.

III. Results

1. MTT assay

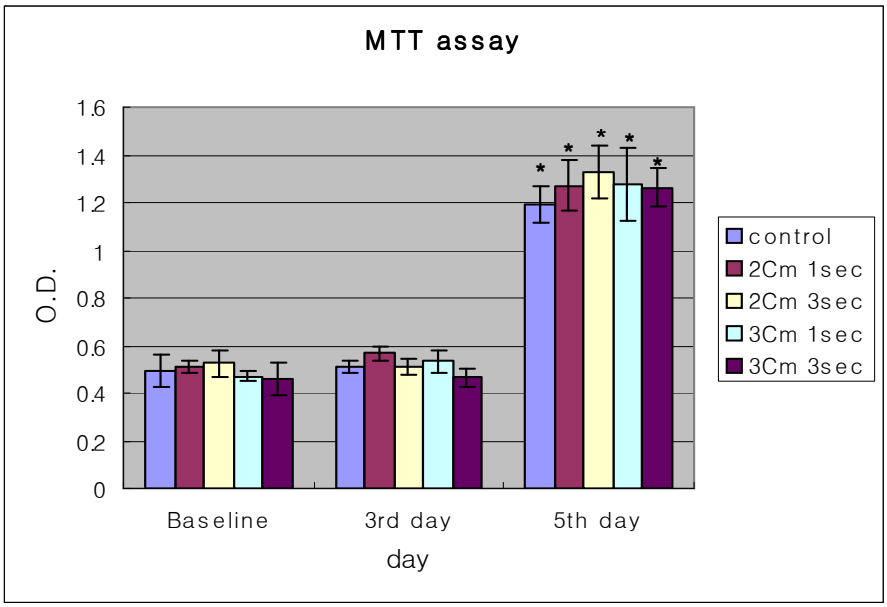
On the 5th day after laser irradiation, statistically significant increase of cells were found compared to the baseline.

At the baseline, 3rd day and 5th day of laser irradiation there were no significant difference of cell increase among the groups. (Table 1, Figure 1)

Table 1. MTT assay (group means ± SD; O.D.: optical density)

	Baseline	3rd day	5th day
control	0.493±0.066	0.510±0.022	1.193† ±0.079
2Cm 1sec	0.511±0.022	0.568±0.029	1.272† ±0.103
2Cm 3sec	0.525±0.054	0.514±0.034	1.328† ±0.114
3Cm 1sec	0.469±0.022	0.533±0.045	1.278† ±0.151
3Cm 3sec	0.458±0.067	0.465±0.037	1.261† ±0.082

† : statistically significant difference compared to baseline($p<0.05$)



* : statistically significant difference compared to baseline($p<0.05$)

Figure 1. MTT assay (O.D.: optical density)

2. ALP detection assay

On the 3rd and 15th day after laser irradiation, ALP positive cells were found in control group.(arrow head) More ALP positive cells were found in laser irradiation groups compared to control group on the 3rd day. When it came to the 15th day, much more ALP positive cells were found in 2cm,1sec group than other groups on the 15th day.(Figures 3,4,5)

3. ALP activity

Statistically significant increase of ALP activity was seen on the 3rd, 5th, 7th, and 10th day after laser irradiation compared to the baseline (Table 2). The control and 3cm,3sec group showed statistically significant decrease of ALP activity between the 3rd and 5th day and no significant increase till the 10th day.

On the other hand 2cm,3sec and 3cm,1sec groups showed statistically significant decrease of ALP activity between the 3rd and 5th day, and significant decrease of ALP activity till the 10th day.

Especially 2cm,1sec group showed increase of ALP activity not statistically significant from the 3rd day to the 10th day.

On the 3rd day after laser irradiation 3cm,1sec and 3cm,3sec group showed statistically significant high score of ALP activity compared to other groups.

On the 5th day after laser irradiation, statistically significant high score of ALP activity was found in experimental groups compared to control group and no statistical difference of ALP activity was found experimental groups.

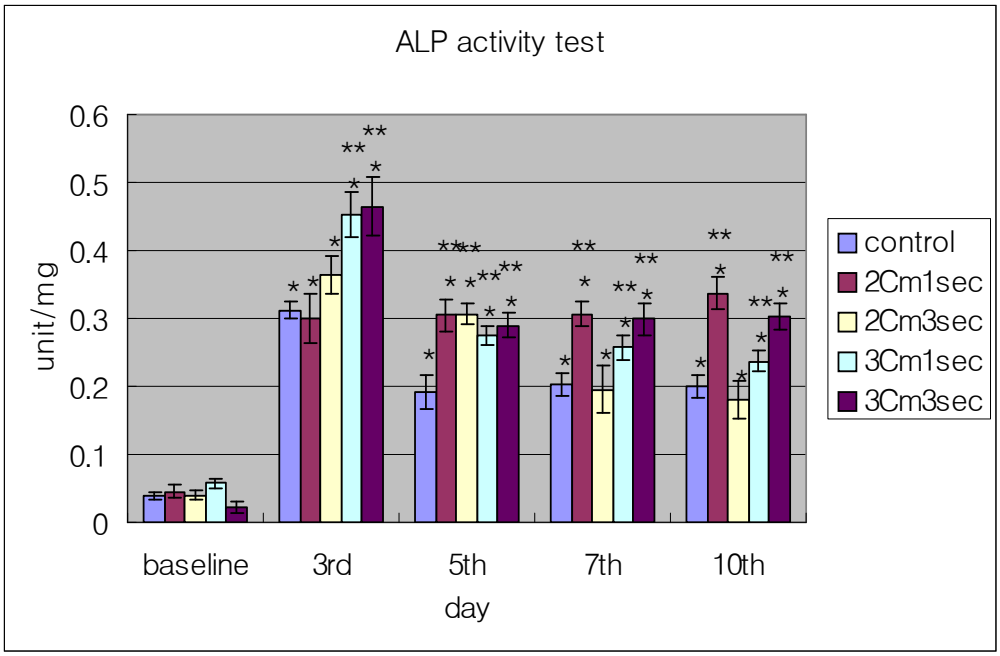
On the 7th and 10th day 3cm,1sec , 3cm,3sec and 2cm,1sec group showed significantly high score of ALP activity compared to 2cm,3sec and control group. And the ALP activity score increased as it went from 3cm,1sec, 3cm,3sec to 2cm,1sec group and there was no

significant difference between 3cm,3sec and 2cm,1sec group.(Table 2, Figure 2)

Table 2. ALP activity (group means ± SD; unit/mg)

	baseline	3rd day	5th day	7th day	10th day
control	0.039±0.006	0.312† ±0.013	0.193† ±0.025	0.202† ±0.017	0.201† ±0.017
2Cm1sec	0.045±0.010	0.300† ±0.035	0.305†‡ ±0.024	0.306†‡ ±0.018	0.337†‡ ±0.024
2Cm3sec	0.039±0.007	0.365† ±0.028	0.306†‡ ±0.015	0.195† ±0.035	0.181† ±0.028
3Cm1sec	0.057±0.008	0.453†‡ ±0.033	0.275†‡ ±0.013	0.257†‡ ±0.018	0.237†‡ ±0.015
3Cm3sec	0.023±0.008	0.465†‡ ±0.044	0.290†‡ ±0.017	0.299†‡ ±0.023	0.302†‡ ±0.020

† : statistically significant difference compared to baseline($p<0.05$)
‡ : statistically significant difference compared to control group($p<0.05$)



*: statistically significant difference compared to baseline ($p<0.05$)
**: statistically significant difference compared to control group($p<0.05$)

Figure 2. ALP activity test (unit / mg)

IV. Discussion

Low-power laser has been widely used in medicine, mostly on pathological tissues presenting any degree of alteration, such as healing or inflammatory processes.[36] We decided to grow the cells in a medium supplemented with 5% concentration of FBS. Serum is an important supplemented for culturing cells,[9] and the best growth will occur using medium containing at least 10% FBS. MTT was performed to observe the growth of PDL cells in this study. And to get the least effect of FBS, medium with 5% FBS was used to see the effect of laser.

Many studies tell us that low power laser irradiation activates growth of various kinds of cells.[2,3,8,18,27,38,39] For example, there is a report that applying laser of $3J/cm^2$ and $4J/cm^2$ to cultured fibroblasts(NIH 3T3 cells) twice every six hours resulted in 3 or 6 times increase of cells compared to non-lasered group.[23]

Laser of 0.5W power was used for 1 second and 3 seconds in this study, thus the energy was $0.5J/cm^2$ and $1.5\ J/cm^2$ respectively. This is less energy than usually applied in other in-vitro study. And this may have caused the cells to over stimulate since power was stronger than 0.1W, which is generally used in-vitro study.

As for the effect of single low-power CO_2 laser irradiation on cell proliferation, both the experimental groups and control group showed statistically significant increase of cells on the 5th day compared th

baseline after laser irradiation and there was no significant difference between experimental group and control group. This means there was a little cell proliferations effect with low-power CO_2 laser in this experimental condition despite different where total energy and power density.

2cm and 3cm irradiation distance results in 116.3%(=W/πr^2), and 58.9% of power density respectively according to 'the relationship between distance of lens and energy density'. The strength of energy transferred to cells depends on power density even when they have the same energy of laser irradiation. Actually 1cm distance irradiation group was involved in this study. But cell necrosis occured because of high energy that reached to PDL cells. So 1cm distance irradiation group could not involve in experiments.

It is not yet well understood how low-power laser irradiation actually affects PDL fibroblast's differentiation to osteoblastic cells. However it is Certain that PDL cell has the ability to differentiate into various kind of cells, that is it can change to cells that makes alveolar bone, depending on given conditions. According to the study of Yuya Murakami et al. in 2003, isolating ALP positive subpopulation is available in PDL fibroblast by immunomagnetic method.[20] In the preliminary study preparing for this experiment, qualitative analysis of ALP was used as a marker of hard tissue formation. The medium containing α-MEM with 10% FBS was used, and ALP positive sample

was obtained from contol group and other groups which were irradiated to low-power lasers by different application methods. (Figures 3,4,5) This shows the pluripotential characteristic of PDL fibroblast mentioned before.

Generally, in order to see ALP activity from PDL cells, materials like 1α,25-dihydroxyvitamin D_3 needs to be added.[4] Various studies have shown that mechanical stress like cyclic stretching decreases the ALP activity.[4,37] So it is necessary to find out what kind of stimulus low-power laser irradiation do to PDL cell differentiation, and what changes the ALP activity and whether there is a difference depending on irradiation method.

In this study, single low-power CO_2 laser irradiation may play positive role of PDL cell differentiation to osteoblastic cells. 2cm,1sec irradiation condition was found to be the most effective condition in this study. And 3cm,3sec was also found to be efficient. Generally, when various factors related to laser irradiation are changed , the results of laser irradiation become different. For example, according to the results of the study by Ueda Y et al. in 2003, low-frequency pulsed laser irradiation significantly stimulates bone formation. And this proved that the pulse frequency of low-power laser irradiation is an important factor affecting biological responses in bone formation.[33] In our study most parameters related to the laser irradiation are constant(e.g., wavelength, power output, irradiation area), except power

densities and related exposure time. Distance between cultured cells and laser tip is related to power density of irradiation. And irradiation time is related to total amount of energy that reach to cells. That is, there is low energy if power density is high, and high energy if power density is low thereby more effects to cell differentiation.

This result shows that among various factors, CO_2 laser irradiation, single or double irradiation, with less energy than 3,4J/cm^2 at which acceleration of PDL cell proliferation or synthesis of collagen takes place, differentiation of PDL cells could be activated.

The right time for laser irradiation to achieve the most efficient effect of low-power laser is not yet cleary defined even though many studies on laser have been carried out. A study using rat calvarial cell reported that cell growth and differentiation were affected by laser irradiation at early stages of cell culture.[22] Though not all cells behaved in the same manner, we could suggest that external stimulus like low-power laser irradiation to cells at early stage of growth or differentiation could greatly influence to growth and differentiation. In addition, this study showed that differentiation of PDL cell to osteoblastic cells was more influences than PDL cell growth was.

Then what controls low-power laser's effect of cell proliferation and differentiation? Most studies regarding the laser effect on cells have reported specific intracellular changes.[10,15,17] Theses changes are mostly related to the calcium metabolism, which would be either affecting its

concentration or intracytoplasmic transport. Such alterations would stimulate cell division, in detriment of cell production, which could explain the reason why the cell growth was altered. However concrete mechanism of laser irradiation to proliferation and differentiation of cells is not clear. We can guess that heat and photostimulation[17,28] given by laser might affect differentiation of cells, and should clarify the effect of laser to differentiation of cells through various kinds of experimental design.

In conclusion, it is not possible to explain proper mechanism. But in this study if single low-power CO_2 laser irradiation is done to human PDL fibroblast, cell proliferation was found in both experimental and control group compared to baseline on the 5th day of laser irradiation. But, no significant differences were found among groups.

In this study, low energy with high power density and high energy with low power density had more effects to cell differentiation. Additionally, statistically significant increase of ALP activity was found in 2cm,1sec and 3cm,3sec groups till the 10th day, and it was found to be the most effective way to PDL cell's differentiation into osteoblastic cells.

V. Conclusion

The aim of this study is to evaluate the effect of low-power CO_2 laser in growth and proliferation of cultured human PDL fibroblast in vitro and to understand better about the effect of laser on periodontal regeneration. The study also tried to find out the most effective degree of energy and power density where cell proliferation and differentiation to osteoblastic cells highly takes place after laser irradiation.

The experimental groups were divided into 4 groups by applying different irradiating distances from cultured cell to laser tip; 2Cm(Focal spot=∅0.74mm), 3Cm(Focal spot=∅1.04mm) and different irradiating time; 1 second, 3 seconds. And they were applied to laser at a power of 0.5W with 50Hz under continuous mode. The control group was not applied to laser. MTT and ALP activity test were performed to observe the growth of PDL cells and cell differentiation to osteoblastic cells. The result are as follows.

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2. Statistically significant increase of ALP activity was seen on the 3rd, 5th, 7th, and 10th day after laser irradiation compared to the baseline.

Among this, there were highest ALP activity on 3rd day except 2cm,1sec group. 2cm,1sec group showed increase of ALP activity not statistically significant from the 3rd day to 10th day.

On the 7th and 10th day 3cm,1sec, 3cm,3sec and 2cm,1sec group showed significant high score of ALP activity compared to 2cm,3sec and control group.

In this study, there was a weak effect of low-power CO_2 laser on cell proliferation but there was a better effect on cell differentiation. 2cm,1sec irradiation was found to be the most effective condition for PDL cell differentiation in this study and 3cm,3sec was found to be efficient.

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Figure Legends

Figure 3. On the baseline after laser irradiation, ALP positive cells were barely seen (light microscope×100).

Figure 4. A: 3rd day control group
B: 3rd day 2cm,1sec group, C: 3rd day 3cm,1sec group
D: 3rd day 2cm,3sec group, E: 3rd day 3cm,3sec group

On the 3rd day after laser irradiation, ALP positive cells were found in control group. (A: arrow head) More ALP positive cells were found in laser irradiation groups compared to control group.(B,C,D,E) (light microscope×100)

Figure 5. A: 15th day control group
B: 15th day 2cm,1sec group, C: 15th day 3cm,1sec group
D: 15th day 2cm,3sec group, E: 15th day 3cm,3sec group

15th day control group (A) showed similar aspect as 3rd day control group.(Figure 4-A) More ALP positive cells were found in experimental groups (B,C,D,E) compared to control group.(A) And much more ALP positive cells were found in 2cm,1sec group (B) among other experimental groups (light microscope×100).

Figure I

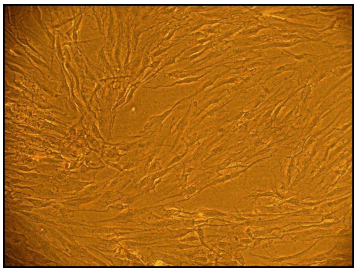

Figure 3

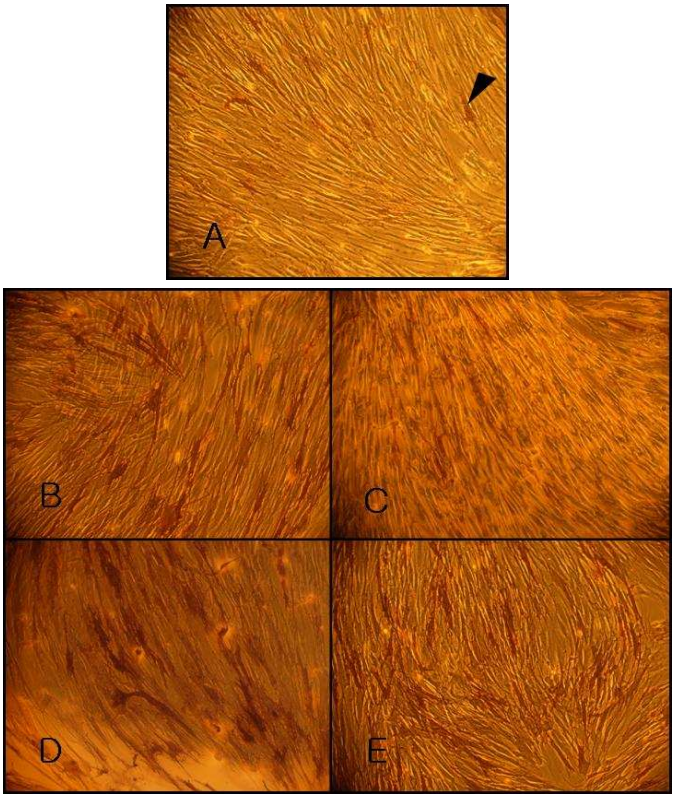

Figure 4

Figure II

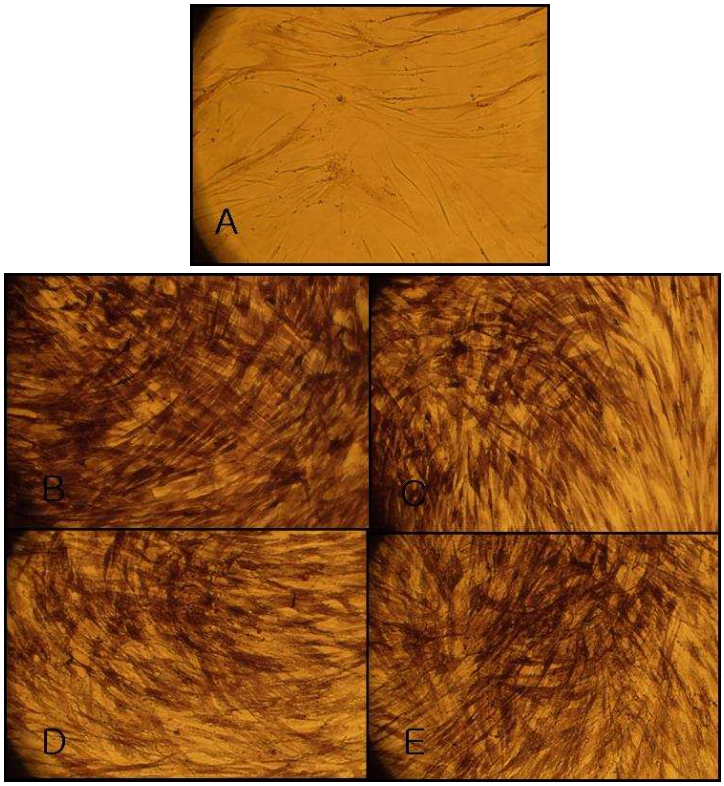

Figure 5

국문 요약

단일조사 저출력 CO_2 Laser가 치주인대 섬유아세포의 세포 증식능과 세포 분화에 미치는 영향.

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장 인 권

이 논문은 단일조사 저출력 CO_2 laser조사가 치주인대 섬유아세포의 증식과 분화에 미치는 영향을 살펴보고 가장 효과적인 에너지와 파워밀도(power density)를 알아보기 위하여 다음과 같이 실험하였다.

0.5W 출력, 10.6 ㎛ 파장, 50 Hz 연속형 CO_2 laser를 사용하여, 실험군은 laser tip과 배양된 세포 사이의 거리를 2cm, 3cm으로 나누고, 조사시간을 1초, 3초로 나누어 4개의 군으로 설정하였고 대조군은 laser를 조사하지 않은 군으로 하였다. 치주인대 섬유아세포의 증식정도와 골모세포로의 분화정도를 보기 위하여 각각 MTT 실험과 ALP activity 실험을 시행하여 다음과 같은 결과를 얻었다.

1. Laser를 조사하고 난 후 5일째에, 모든 군에서 유의하게 세포가 증식되는 것을 확인할 수 있었고 조사방법간에 유의한 차이가 없었다.

2. 대조군과 실험군에서 0일째에 비하여 3일째, 5일째, 7일째, 10일째에 통계적으로 유의하게 ALP activity가 증가하였고, 이중 2cm,1sec 군을 제외하면 3일째에서 가장 높은 ALP activity 값을 보였다. 특징적으로 2Cm,1sec 군은 3일째부터 10일까지 통계적으로 유의하지는 않지만 시간이 지남에 따라서 ALP activity가 증가함을 보였다.

7일과 10일째에는 2cm,1sec, 3cm,3sec군에서 다른 군에 비하여 큰 activity값을 보였다.

이번실험에서 저출력 CO_2 laser 조사는 세포의 증식보다는 분화에 더 큰 영향을 끼쳤고, 2cm,1sec, 3cm,3sec군이 치주인대 섬유아세포의 분화에 가장 효과적인 laser 조사방법으로 분석되었다.

핵심되는 말: CO_2 laser, 치주인대 섬유아세포, 세포증식, 세포분화, 파워밀도, 에너지.