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In Vitro Effect of 808-nm Diode Laser on Proliferation and Glycosaminoglycan Synthesis of Rabbit Articular Chondrocytes

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Abstract : The aim of the study was to assess the in vitro effect of 808-nm InGaAs diode laser on rabbit articular chondrocyte proliferation and sulphated glycosaminoglycan (sGAG) synthesis in alginate bead. Previous studies revealed either positive or negative stimulatory effects of laser on different types of cells. A 808-nm InGaAs diode laser at 1.0 W power output was used to irradiate the rabbit chondrocytes in alginate beads with energy densities of 31 J/cm^2 (G 1) and 62 J/cm^2 (G 2) corresponding to the experimental groups for 10 seconds and 20 seconds, respectively at 24, 48, 72 and 96 hours after seeding. Control group was left untreated. MTT assay was performed at 1 week and 2 weeks after the 1st laser irradiation in alginate beads. sGAG synthesis in alginate beads at 1 week and 2 weeks were determined by DMMB assay. Histological evaluation for cellular distribution and sGAG deposition around the cells were performed by alcian blue stain. MTT assay revealed no positive stimulatory effect in cell proliferation in alginate bead. DMMB assay results showed significantly increased sGAG production in G 2 chondrocytes at 2 weeks. Image analysis of alcian blue stained slides also showed significantly higher percentage of positive alcian blue stain in G 2 chondrocytes. This result suggests that 808-nm InGaAs diode laser with 1.0 W power output although cannot stimulate cell proliferation it can increase the cell secretion activity and sGAG deposition in alginate beads.

Key words **:** Diode/diode laser, Orthopaedic, Tissue regeneration and healing.

Introduction

Osteoarthritis (OA), a degenerative joint disease, mostly affects the larger and weight bearing joints such as the knee (2). Various treatment methods have been implemented since decades to treat OA including weight control and using non-steroidal anti-inflammatory drugs (NSAIDs), steroids, immunosuppressants and synovectomy (11,20,26). Low-level laser therapy (LLLT) is widely used in wound healing for the last three decades (17). The application of LLLT in tissue regeneration and dentistry is also common, where it is used to heal the wound (8). Clinical studies have shown the analgesic and wound healing efficacy of LLLT. LLLT, a non-invasive therapy, has become a part of medicine and physiotherapy for treating the rheumatoid arthritis and OA to control the joint pain and thus improving the joint motion (4).

LLLT is a non-thermal irradiation with a wavelength between visible light and the near-infrared range. During irradiation, the cells absorb the light which can initiate the signaling cascades leading to various biological effects such as cell growth, proliferation, collagen synthesis and differentiation (30). It was also reported that LLLT could increase the sulphated glycosaminoglycans (sGAG) synthesis in rabbit chondrocytes. The effects of LLLT on the biological tissue depend on the specification of light source such as wavelength, output power, energy density and also tissue structure (12). Low-levels of red or near infrared laser can prevent apoptosis of cells and improve cell proliferation (10).

LLLT has been widely used in bone fracture healing and meniscus damage repair (15,23,27). In cartilage damage, application of LLLT is not yet fully established. Although several new techniques have been implemented in articular cartilage tissue regeneration, their applications have not yet been proved to be completely successful (29). The biostimulatory effects of LLLT in cartilage can be a great interest of the researcher but before clinical application, it is necessary to test the biostimulatory effect of LLLT both in vitro and in vivo (12).

Alginate has been commonly used for chondrocyte culture. Many scientists studied alginate extensively for the application in cartilage tissue engineering as a matrix for cellular encapsulation and culture. Alginate may provide the facilities of uniform distribution of cells as well as it prevents the cells from floating out. Alginate can help to retain the chondrocyte-specific phenotype (14,19). It has been reported that synthesis of chondrocyte matrix can be the result of alginate or its degradation products (16).

The aim of this study was to evaluate the biostimulatory effect of 808-nm indium-gallium-arsenide (InGaAs) diode laser on the proliferation and glycosaminoglycan (sGAG) production of rabbit articular chondrocytes both in monolayer and alginate culture system.

Materials and Methods

Isolation and culture of rabbit chondrocytes

Rabbit chondrocytes were isolated from six New Zealand

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white rabbit. The animals were consulted in accordance with "Guide for care and use of Laboratory Animals" of Chungbuk National University. Full thickness articular cartilages were aseptically collected from the distal femoral condyle and digested with collagenase type I (0.1%) (Welgene, Daegu, South Korea) for 8 h at 37°C in a shaking water bath. After isolation, the cells were cultured with high glucose DMEM (Welgene, Daegu, South Korea) containing 10% FBS (Welgene, Daegu, South Korea) at 37°C in 95% air and 5% CO_2 humidified atmosphere. Culture medium was changed two times per week. After the cells were confluent, they were trypsinized with 0.25% trypsin-EDTA (Welgene, Daegu, South Korea) and sub-cultured furthermore. Passage 2 cells were used in this experiment.

Encapsulation of rabbit chondrocytes in alginate bead

Cells were resuspended in 1 mL after centrifugation in a 50 mL conical tube. Cell suspension was then mixed with an equal volume of sodium alginate (2.4% in 0.15 M NaCl, filter-sterilized by 0.45 μm filter) to get the final alginate concentration of 1.2%. We used 5×10^6 cells/mL to make the beads. Alginate-cell suspension was then slowly dispensed through a modified yellow tip drop wise into a 102 mM $CaCl_2$ gelation solution (pH 7.4). Each drop contains approximately 30 μL cell suspension to make each bead. The beads were polymerized in gelation solution for 15 min at room temperature. After completion of polymerization, calcium chloride solution was removed. The newly formed beads were then washed three times with 0.15 M NaCl, followed by two washes with fresh DMEM. Alginate beads were then transferred into six well plate with DMEM containing 10% FBS and cultured at 37°C in a 5% CO_2 humidified atmosphere for 2 weeks. Medium was changed three times a week. Beads were transferred to a new six well plate every week to prevent the formation of cell monolayer at the bottom. Samples were harvested at 1 and 2 weeks.

Cell proliferation assay in alginate bead culture

Proliferation of the rabbit chondrocytes in alginate beads was determined using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MO, USA) colorimetric assay. The rabbit chondrocytes in alginate beads were grouped as control, G 1 and G 2. The alginate-cell constructs were incubated for 24 hours in 5% CO_2 humidified atmosphere at 37°C. After 24 hours of seeding cells into alginate beads, a 808-nm InGaAs diode laser (DVL-20 Diode Laser System®, Aasuka Medical Inc., Kyoto, Japan) was used to irradiate the chondrocytes. Laser light was delivered through a 400 μm fiber optic system with 1.0 W power output in the continuous wave mode. The fiber tip was perpendicularly contacted at the bottom of well and each bead was irradiated for 10 and 20 seconds in G 1 (31 J/cm^2) and G 2 (62 J/cm^2) (energy density = J/irradiated area) group, respectively. The control beads were left untreated. Laser was irradiated at 24, 48, 72 and 96 hours after seeding of the cells. Medium was changed every three days. MTT assay was performed at 1 week and 2 weeks after the 1st irradiation. Briefly, one bead from each group was taken into an eppendorf tube. 800 μL of DMEM (fresh) and 80 μL of MTT stock solution (5 mg/mL) were added to the bead. The bead was then incubated at 37°C for 4 hours. After 4 hours, the medium was removed and 1 mL of DMSO (Junsei Chemical Co. Ltd, Japan) was added and incubated for 2 hours in order to dissolve the formazan crystals completely and was transferred to 96 well plate at 200 μL per well with a total number of 4 wells in each group. The absorbance was read using a spectrophotometer (EMax®, Molecular Devices, CA, USA) at 570 nm. The OD values were presented as mean ± standard deviation (SD). MTT assay in alginate beads were done in triplicate.

Sulphated glycosaminoglycan (sGAG) production assay

Dimethyle methylene blue (DMMB) assay was performed at 1 week and 2 weeks after the 1st laser irradiation in order to determine the sGAG. A solubilization solution containing 55 mM sodium citrate, 5 mM EDTA, 0.15 M NaCl, 5 mM cysteine hydrochloride and 0.56 units/mL papain (Sigma-Aldrich, St Louis, MO, USA) was used to solubilize three alginate beads. After complete solubilization, the samples were incubated at 60°C for 24 hours and then centrifuged at × 300 g for 3 minutes. 10 μL of sample was added to 190 μL of DMMB dye (Sigma-Aldrich, St Louis, MO, USA) solution (pH 2.00) and the metachromatic reaction was determined using a spectrophotometer at an absorbance of 540 nm to determine the sGAG deposited. At each time point of DMMB assay, data were collected from 6 wells in each group and all assays were done in triplicate. A standard curve was prepared using chondroitin 6 sulphate (Sigma-Aldrich, St Louis, MO, USA) to determine the sGAG (μg/mL).

Histological evaluation

Beads from each group were harvested at 1 week and 2 weeks after the 1st laser irradiation and fixed in 10% formalin at room temperature for overnight. After rinsing with PBS and serial dehydration, beads were paraffin embedded and sectioned at 5 μm thickness. Cut sections were then placed on glass slides, dried overnight, deparaffinized in xylene, rehydrated through graded alcohol and stained with Alcian blue (Sigma-Aldrich, St Louis, MO, USA). The alcian blue stained slides were observed in the microscope (Damisystem, Masan, South Korea) to evaluate the distribution of cells in alginate beads. The Alcian blue stained bead sections were also analyzed by ImageJ® software 1.46r (Wayne Rasband, National Institute of Health, USA) from five different focuses in a bead to determine the percentage of blue color deposited around the cells. Image analysis was performed in triplicate. The higher the percentage, the higher was the amount of sGAG deposition.

Statistical analysis

Data were evaluated as the mean ± SD. Statistical significance of mean difference among the groups was analyzed by one-way analysis of variance (ANOVA) with Tukey's test using the SPSS® 20 statistical software (SPSS Inc., Chicago, USA). Statistical significance was considered when the P value was < 0.05.

Results

Cell proliferation

MTT cell proliferation assay revealed that InGaAs laser irradiated cultures of both groups (G 1 and G 2) did not increase or decrease the cell proliferation significantly ($P < 0.05$) in comparison with the non-irradiated control during 1 week and 2 weeks after the 1st laser irradiation (Fig 1). In fact, cells were proliferating in alginate bead in a similar pattern in control, G 1 and G 2 throughout the culture period of 2 weeks.

Sulphated glycosaminoglycan (sGAG) production

DMMB assay showed no significant increase ($P < 0.05$) of sGAG production in alginate beads of both experimental groups as compared to the control during 1 week culture period, but G 2 chondrocytes showed significantly higher amount of sGAG synthesis compared to both control and G 1 chondrocytes during 2 weeks after the 1st laser irradiation. G 1 chondrocytes also showed higher amount sGAG synthesis than control, although it was not significant (Fig 2).

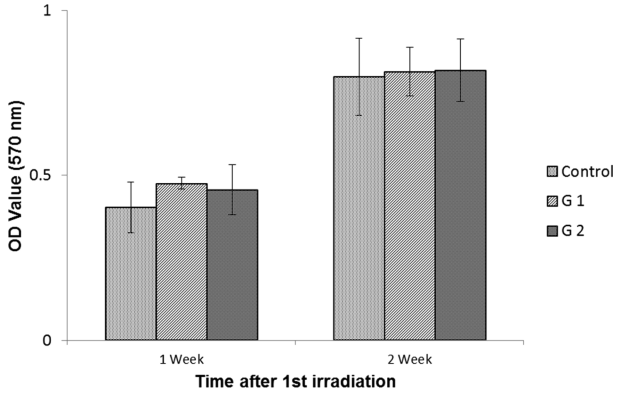

Fig 1. Cell proliferation test in alginate beads using MTT assay. Data were presented as mean ± SD. No significant difference in cell proliferation was found among the groups during 1week and 2 weeks culture period.

Histological evaluation

Rabbit chondrocytes cultured in alginate beads in all groups showed a round morphology during the culture period. Cells were found to have homogenous distribution within the beads in all groups. Image analysis of alcian blue stained rabbit chondrocytes in alginate beads revealed a significantly higher percentage of positive alcian blue stain in G 2 chondrocytes than control and G 1 chondrocytes at 2 weeks after the 1st laser irradiation. However, no significant difference was found among the groups at 1 week after the 1st laser irradiation (Fig 3, 4). This image analysis result revealed the higher amount of sGAG deposition around the cells in G 2 than other groups.

Discussion

The effects of LLLT have been studied in cell growth, proliferation (31) and differentiation (3,5). Various types of cell

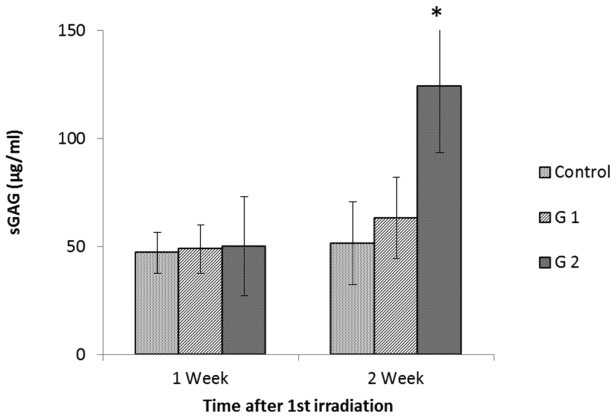

Fig 2. Determination of sGAG using DMMB assay. G 2 chondrocytes synthesized significantly higher sGAG than G 1 and control at 2 weeks of culture period. Although G 1 chondrocytes showed higher sGAG synthesis compared to control at 2 weeks, it was not significant. $P < 0.05$ was considered as significant. The asterisk mark indicates the point in time where significant difference presents.

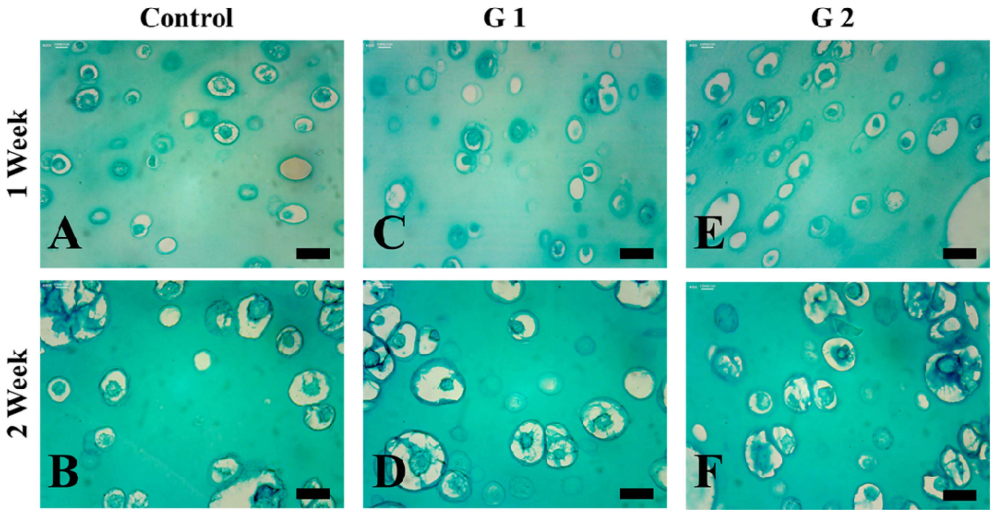

Fig 3. Deposition of glycosaminoglycan in alginate beads. A-B: Control; C-D: G 1; E-F: G 2. G 2 chondrocytes showed higher sGAG production than G 1 and control chondrocytes at 2 weeks after the 1st laser irradiation. Alcian blue stain; magnification: 400 ×.

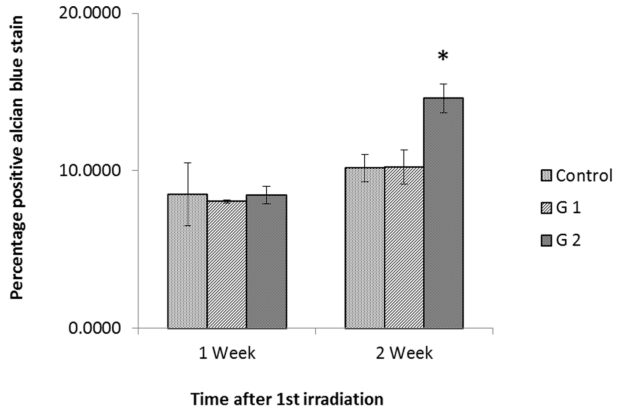

Fig 4. Image analysis graph showing the percentage of positive alcian blue stain. After 2 weeks of 1st laser irradiation, it was found to have significantly ($P < 0.05$) higher percentage of positive alcian blue stained area in G 2 chondrocytes than G 1 and control chondrocytes. The higher the percentage, the higher will be the amount of sGAG deposited around the chondrocytes.

such as fibroblasts, endothelial cells, skeletal cells, keratinocytes, myoblasts and other types have been used to investigate in vitro cell proliferation effect of LLLT (3,5,12,24,28, 31). In this study, we investigated the effect of 808-nm InGaAs diode laser on the proliferation and sGAG synthesis of rabbit articular chondrocytes cultured in alginate beads. LLLT acts in the way that it influences the photoreceptors in the cells and converts the cellular chemical energy into the ATP, thus increasing the cell functions and cell proliferation rates (21,22). All these studies mentioned above revealed the positive biostimulatory effect of LLLT on various types of cells. However, in this study, the effect of LLLT in cell proliferation showed no significant stimulation. Cell proliferation was investigated in 3-D culture system in alginate beads and determined by MTT assay. 3-D culture system was failed to prove either positive or, negative effect of LLLT on cells; rather it revealed that stimulatory effect of LLLT in treated chondrocytes remained similar when compared to the non-treated chondrocytes. Similar result was found for up to 96 hours after the 1st laser irradiation in monolayer culture of rabbit chondrocytes which were irradiated at the same energy densities per well in a similar pattern (data not shown). Several factors such as laser beam characteristics and spreading, power output, calibration of the equipment should be considered to investigate the effect of laser on cells (7). The result of cell proliferation effect of LLLT in this study is also supported by other authors when it has been reported that GaAlAs lasers with 830-nm of wavelength was failed to enhance the proliferation of cultured fibroblasts and keratinocytes (22). He-Ne laser was found to increase the cellular motility of keratocyte but failed to stimulate cell proliferation (9).

In the current study, we irradiated the rabbit chondrocytes in alginate beads with InGaAs laser for a period of 4 days after 24 h of seeding the cells. The frequency of the laser used to irradiate the cells can also affect cellular proliferation (30). Osteosarcoma cells irradiated with single dose or daily irradiation dose of different energy by 830-nm GaAlAs laser showed no significant difference in cell count or MTT activity when compared to the untreated control groups over a period of 10 days (6).

Cell proliferation effect of LLLT is influenced by the wavelength of the laser and the type of cell irradiated (18). The magnitude of the LLLT on the proliferation of cell may vary due to the physiological state of the cells during irradiation period (1). Poorly growing cells during the irradiation period can be stimulated effectively by the laser than the fully functional cells or cells growing in 10% serum-rich environment, because there remains nothing to stimulate by LLLT (25).

One of the aims of this study was to investigate sGAG synthesis in alginate beads determined by DMMB assay and alcian blue staining. Both DMMB and histological alcian blue stain results revealed significantly increased sGAG synthesis by G 2 chondrocytes than G 1 and non-treated control chondrocytes at 2 weeks after the 1st laser irradiation. Similar results were also reported in rabbit articular chondrocytes when irradiated with He-Ne laser and determined by toluidine blue staining technique. Increased synthesis of sGAG results probably due to that when LLLT is absorbed by the light acceptor remaining in the cells which may stimulate the intracellular signal transmission system and thus increasing the gene expression and cellular metabolism by magnifying the signal to achieve the biostimulatory effect on cells (12).

Several reports suggested that laser might have biostimulatory effect when irradiated with 0.05 to 10 J/cm^2 and bioinhibitory effects might have resulted with energy density above 10 J/cm^2 (13,17). However, we did not reveal any bioinhibitory effect of laser at a high energy density of 31 J/cm^2 and 62 J/cm^2.

In conclusion, our results in this study suggest that 808-nm InGaAs diode laser with 1.0 W power output at high energy density although cannot stimulate cell proliferation, it has no bioinhibitory effects on cellular function and metabolism; instead it can increase the cell secretion activity and sGAG deposition.

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토끼 관절 연골세포의 증식과 글리코스아미노글리칸 합성에 대한 808-nm 다이오드 레이저의 효능 평가

Maruf Minar · 황야원 · 최석화 · 김근형[1]

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요 약 : 본 연구의 목적은 알지네이트 비드에서 배양한 토끼의 관절 연골세포의 증식과 황산화 글리코스아미노글리칸 합성에 대한 808-nm InGaAs 다이오드 레이저의 효과를 확인하는 것이다. 이전의 연구들에서 서로 다른 종류의 세포에서 레이저의 양성 또는 음성 자극 효과가 알려졌다. 알지네이트 비드 내의 토끼 연골세포에 1.0 W 세기의 808-nm InGaAs 다이오드 레이저가 31 J/cm^2 (1 그룹), 62 J/cm^2 (2 그룹)의 에너지 밀도로 상응하는 그룹에 10초, 20초 동안 24, 48, 72, 96시간째에 각각 조사되었다. 대조군은 처리하지 않았다. 1차 레이저 조사 1주, 2주 후에 MTT 분석이 실시되었다. 황산화 글리코스아미노글리칸 합성은 DMMB 분석을 통해 평가되었다. 조직학적 평가를 위한 세포의 분포와 세포 주변의 황산화 글리코스아미노글리칸 침착은 알시안 블루 염색을 통해 평가되었다. MTT 분석을 통해 알지네이트 비드에서 세포 증식에는 양성 자극 효과가 없음을 알 수 있었다. DMMB 분석을 통해서 2 그룹의 2주차 연골세포에서 황산화 글리코스아미노글리칸 생성이 특이적으로 증가했음을 알 수 있었다. 알시안 블루 염색상에서도 2 그룹 연골세포에서 양성 염색상이 특이적으로 많은 비율을 차지함을 알 수 있었다. 본 연구를 통해 1.0 W 세기의 808-nm InGaAs 다이오드 레이저가 연골세포 증식에 영향이 없으나 알지네이트 비드에서 세포 분비 활동을 자극하여 황산화 글리코스아미노글리칸 침착을 증가시킬 수 있음을 확인하였다.

주요어 : 다이오드/다이오드 레이저, 정형외과, 조직재생과 치유