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Research Report

# The effects of different 650 nm laser diode irradiation times on the viability and proliferation of human periodontal ligament fibroblast cells

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## *ABSTRACT*

***Background:*** *Endo-perio lesions are clinical manifestations of inflammation in the periodontal and pulp tissue. Damage to the periodontal ligament can inhibit its ability to regenerate. Therefore, laser therapy use is expected to improve the prognosis with regard to healing lesions. Unfortunately, the duration of irradiation during laser diode therapy can influence the viability and proliferation of human periodontal ligament fibroblast (hPDLF) cells.* ***Purpose:*** *This study aims to determine the effects of different irradiation exposure times of the 650 nm laser diode of the pulsed mode type on the viability and proliferation of human periodontal ligament fibroblast cells.* ***Methods:*** *This study constituted a laboratory experiment on hPDLF cells using 650 nm laser diode irradiation. Six groups formed the research subjects in this study, namely; two control groups, two radiation groups respectively subjected to irradiation exposure of 15 seconds and 35 seconds duration followed by 24-hour incubation, and two radiation groups exposed to irradiation for 15 and 35 seconds respectively followed by 72-hour incubation period. The viability and proliferation of those cells were subsequently calculated by ELISA reader, while the data was analyzed by means of one-way ANOVA and Tukey tests.* ***Results:*** *The significance value of the viability scores between the 15-second irradiation group and the 35-second irradiation group was less than 0.05, indicating that there was a significant difference between these treatment groups. Similarly, the significance value of proliferation scores between the 15-second irradiation group and the 35-second irradiation group was less than 0.05, again indicating a significant difference between these treatment groups.* ***Conclusion:*** *Irradiation using a 650 nm laser diode 15 seconds and 35 seconds in duration can induce an increase in the viability and proliferation of hPDLF cells.*

***Keywords:*** *cell proliferation; cell viability; human periodontal ligament fibroblast cells; laser diode*

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## INTRODUCTION

Periodontal tissue and dental pulp actually have a very close relationship since they are derived from ectomesenchymal tissue. Endo-perio lesions are clinical manifestations of inflammation that occur due to the relationship between periodontal and pulp tissues.[1] Moreover, endo-perio lesions can undergo root canal treatment.[2] Unsuccessful conventional root canal treatment is usually due to complex root canal anatomy rendering the removal of all healthy pulp tissue and bacterial residue difficult.[3] Since damage to the periodontal ligament limits its regenerative ability, root canal treatment and laser therapy can improve the prognosis of the lesion healing process.[4]

Progenitor cells are identified in periodontal structures including periodontal ligaments. These cells can differentiate into fibroblasts, osteoblasts, and cementoblasts which play a crucial role in healing periodontal tissue.[5] As dominant cells, fibroblasts are found in connective tissue where they secrete collagen fibers and extracellular substances. Fibroblast cells can also be used to see viability and proliferation.

Human primary fibroblasts derived from periodontal ligaments also play an important role in the development,

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functioning, and regeneration of periodontal tissue.[6,7] Human periodontal ligament fibroblasts (hPDLF) are the main cell types in the tooth-supporting tissue. Fibroblasts, the most common connective tissue cells found in the pulp and periodontal ligaments, produce collagen fibers that actively participate in the healing process.[8] Fibroblasts also function as defense cells because of their capacity to differentiate into odontoblasts and osteoblasts during the healing process. The ability of fibroblast cells to develop rapidly into wound tissue or survive independently accounts for the ease with which they can be cultured for use in biological research.[9]

Light amplification by stimulated emission of radiation (LASER) is a device that produces coherent electromagnetic radiation that has been employed in the field of dentistry since 1960.[10] Lasers are known to induce biphasic dose responses (BDR), namely; biostimulation and bioinhibition responses.[11] This effect is related to an increase in adenosine triphosphate (ATP) in the mitochondria that produce deoxyribonucleic acid (DNA) and ribose nucleic acid (RNA) and whose synthesis increases after laser diode irradiation. This increase can cause cellular responses to injury through the production of proteins associated with repair and healing processes.[12]

Low-level lasers operate within several parameters, including: wavelength, energy source, energy density (fluence), potential density (irradiance), irradiation time, and laser light emission area. A previous study stated that laser light plays a role in increasing fibroblast proliferation.[13] Furthermore, the wavelength of red light emitting diodes (LEDs) light is 600-700 nm. The wavelength of laser therapy is known to have an influence on cell culture, with one previous study even stating that wavelengths of 600-700 nm can stimulate an increase in cell proliferation and differentiation.[5] Another investigation using low-level laser therapy (LLLT) at a wavelength of 650 nm argued that the light of photons received by a cell chromophore can serve to regenerate tissue, reduce inflammation, and reduce pain.[14] Similarly, a proliferation test on fibroblast cells in periodontal ligaments with irradiation times of 16 seconds and 33 seconds reportedly produced an increase in the number of fibroblast cells.[5]

In addition, laser irradiation is divided into two types, namely; continuous mode and pulsed mode. The latter has the advantage that a delay in the irradiation time of the "quench period" causes a decrease in temperature on the surface of the tissue during laser exposure. The use of pulsed mode also allows higher energy consumption than the use of continuous mode.[15]

Consequently, this study aims to determine the effects of irradiation by a 650 nm laser diode using pulsed modes of 15 seconds and 35 seconds' duration on the viability and proliferation of hPDLF cells. The significance of this study is to determine the method and irradiation time of laser diode therapy at a wavelength of 650 nm in root canal treatment in order to achieve optimal improvement in the treatment of periapical lesions.

## MATERIALS AND METHODS

The hPDLF cell culture was produced at the Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta. This study was approved by the Faculty of Dentistry, Airlangga University through the issuing of Ethical Clearence Certificate No. 135/HRECC.FODM/IV/2019. The production of the hPDLF cell culture was subsequently approved by patients who had undergone dental extraction for orthodontic reasons at Professor Soedomo Dental and Oral Hospital, Yogyakarta.

Following initiation of the cell harvesting phase, the first maxillary premolar extracted was inserted into a 10ml tube containing 10% Dulbecco's Modified Eagle's Medium (DMEM, D16171, Sigma-Aldrich Pte. Ltd, Singapore) to which 0.5µg/ml Fungizone (Amphotericin B, GibcoTM Fungizone Antimycotic, Fisher Scientific GSA) and Penstrep 2% (GibcoTM Streptomicin, Fisher Scientific GSA) was added.[16] These teeth were removed from the tube, washed three times with buffered saline phosphate (PBS, P7059 Sigma-Aldrich Pte. Ltd, Singapore) and placed on a petri dish containing a fetal bovine serum medium (FBS 10%, F4135, Sigma-Aldrich Pte. Ltd, Singapore). The teeth were subsequently scalped in the 1/3 apical section, placed on a small petri dish covered with a sterile glass deck, added to a complete medium of 3ml of DMEM 10%, and placed in a large petri dish to be incubated in a $CO_2$ incubator (MEMMERT, INC108Med, Germany). The medium in the petri dish was removed and replaced once every three days.[17] The cells were observed until 80% confluent had been achieved, the periodontal tissues were then removed, and the medium was washed with PBS.[18]

During the secondary culture stage, 2ml of trypsin-EDTA 0.25% (SM-2003-C Sigma-Aldrich Pte. Ltd, Singapore) was added to the medium. If the cells appeared to have been released, the medium would be deposited by pipette into a 5ml tube. Centrifuging was carried out for ten minutes at a speed of 1500 rpm at which point the supernatant was discarded and 1ml of complete medium of DMEM 10% was added until it was homogenized. The cells were then placed in several petri dishes and incubated in a $CO_2$ incubator. The medium was discarded and replaced with new medium once every three days. During subsequent observation under a microscope, if cell confluent reached 80%, calculation of the number of cells would be undertaken.[18]

During the cell treatment stage, the cells were divided into 96 well plates. 100 µl of cell suspension at a density of 2 x 104 cells/well was added to each plate and allowed to stand for two hours. The laser diode light (FNRdentolaser 650 nm) was then irradiated for 15 seconds and 35 seconds at a distance of 1 cm withan output power of 20 mW.[5] In this study, three research groups were formed for viability, namely; a control group, a 15-second irradiation group (incubated for 24 hours) and a 35-second irradiation group (incubated for 24 hours). There were also three research groups for proliferation, namely; a control group, a 15-

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second irradiation group (incubated for 72 hours), and a 35-second irradiation group (incubated for 72 hours).

These cells were incubated in a $CO_2$ incubator for four hours in order that they were reattached after harvest. Four hours later, observations were carried out under a microscope and photographs were taken. The medium on the plate was then discarded by means of a pipette. 100µl of PBS was added to all well cells before itself being disposed of. At this point, complete media was added to each plate containing cells and incubated in a $CO_2$ incubator with a $CO_2$ level of 5% at a temperature of 37° C and a humidity level of 98% for a period of 24 hours (for the purposes of viability) and another period of 72 hours (to induce proliferation).

Observation was conducted under a microscope and photographs taken to determine the viability and proliferation of hPDLF cells. The cell complete culture medium was removed and washed with PBS which was then disposed of. 100µl of MTT was added [5 mg MTT (CT01-5 Sigma-Aldrich Pte. Ltd, Singapore) to each well, together with 1ml of PBS and 9ml of complete DMEM medium. It was then incubated for four hours until formazan was formed. 100µl SDS of 10% stopper solution (RABSTOP1 Sigma-Aldrich Pte. Ltd, Singapore) was added to 0.01 NHCl (Titripur® Sigma-Aldreich Pte. LTD, Singapore) in each well and incubated overnight.[18]

Finally, the number of hPDLF cells was calculated with Elisa Reader (Reader Type: Model 680 XR, Benchmark) at a wavelength of 550nm and a temperature of 25.1°C using Endpoint (fast read) reading type with a mix time of 0 sec. The six plates were alternately inserted into the Elisa Reader, in other words; plates 1-3 for viability (flat-shaped cells with oval nuclei) and plates 4-6 for proliferation (large, flat-branched cells). The living hPDLF cells were colored purplish blue, in contrast to the dead cells which did not display this color. The data was subsequently analyzed statistically with one-way ANOVA and Brown-Forsythe tests to compare more than two groups. The data was also subjected to analysis by a Tukey HSD test in order to compare all treatment group pairs. The statistical analysis undertaken employed SPSS version 20 (IBM, Armonk, New York, USA) statistical software.

## RESULTS

The results of a Brown-Forsythe test, as illustrated by Table 1, showed a significant difference in the average viability scores of the 15-second irradiation group and the 35-second irradiation group with a p-value of <0.05. Similarly, based on the ANOVA test results, as shown in Table 1, a significant difference existed in the average proliferation scores between the 15-second irradiation group and the 35-second irradiation group with a p-value of <0.05.

Moreover, the Tukey HSD test results, as shown in Table 2, indicated a significant difference in the average viability scores between the 15-second irradiation group and the 35-second irradiation group with a p-value of <0.05. Similarly, as shown in Table 2, according to the Tukey HSD test results, there was a significant difference in the average proliferation scores between the 15-second irradiation group and the 35-second irradiation group with a p-value of <0.05.

**Table 1**. The mean and standard deviation scores of the viability and proliferation of hPDLF (OD) cells

| Treatment | n | $\bar{x}$ ± SD | | P |
|---|---|---|---|---|
| | | Viability | Proliferation | |
| Control | 5 | 0.712 ± 0.013 | 0.699 ± 0.009 | |
| P 15’ | 5 | 0.822 ± 0.03 | 0.815 ± 0.019 | 0.000 < 0.05 |
| P 35’ | 5 | 0.936 ± 0.009 | 0.921 ± 0.02 | 0.000 < 0.05 |

Note: $\bar{x}$ = Mean, P = Probability, SD = Standard Deviation, P15’= 15 second irradiation, n = Replication, P35’= 35 second irradiation

**Table 2**. The results of Tukey HSD test of the viability and proliferation of hPDLF Cells

| (I) Treatment groups | (J) Treatment groups | P | |
|---|---|---|---|
| | | Viability | Proliferation |
| Control | P 15’ | 0.000 | 0.000 |
| | P 35’ | 0.000 | 0.000 |
| P 15’ | Control | 0.000 | 0.000 |
| | P 35’ | 0.000 | 0.000 |
| P 35’ | Control | 0.000 | 0.000 |
| | P 15’ | 0.000 | 0.000 |

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## DISCUSSION

In this research, hPDLF cells were employed since in several previous studies the use of such cells produced significant results. In addition to fibroblast cells, osteoblast cells were also involved in bone formation and regulation. In a previous study, a 940 nm laser diode used on osteoblast cells was shown to be capable of triggering the release of autocrine factors, such as TGF-β1 in response to irradiation, but the results were insignificant.[19]

Furthermore, the viability of hPDLF cells indicated their number living in a culture medium, where viability after irradiation with a 570 nm low-level laser was observed at six hours and 24 hours.[13] Low-level laser therapy (LLLT) on stem cells from human exfoliated deciduous teeth (SHED) at a wavelength of 660nm generated the highest cell viability 24 hours after irradiation. On the other hand, the proliferation of hPDLF cells indicated the number of such cells that grow and divide in a culture medium. LLLT at a wavelength of 660 nm on SHED teeth generated the highest cell proliferation which occurred 72 hours after irradiation.[20]

A previous study of a fibroblast cell proliferation test conducted on periodontal ligaments with irradiation times of 16 seconds and 33 seconds and a wavelength of 660 nm showed a significant increase in the number of fibroblast cells.[5] Another previous study using low-level laser diode (λ = 680) suggested that diode lasers be used as an alternative to biomodulation. This effect is related to the increase in ATP in the mitochondria which causes an increase in DNA and RNA synthesis after irradiation with a laser diode. This increase can cause cellular responses to injury through the production of proteins associated with processes of repair and healing.[12]

In addition, pulsed mode has the advantage of there being a delay in irradiation time during the "quench period" which causes a decrease in the surface temperature of the tissue during laser exposure. Moreover, six out of nine previous studies using LLLT show that the use of pulsed mode is more effective than continuous mode.[15] The laser light power dose per laser area (density, unit J/ cm2) is the total laser power (laser power multiplied by the length of exposure time) divided by the total area of the laser. This determines the duration of the laser light exposure which is adjusted to the power dose and the quantum yield.[21] Therefore, this study employed the pulsed mode irradiation method.

A previous study using 650nm LLLT argued that released photon light can be absorbed by the cell chromophore to regenerate tissue, reduce inflammation, and ease pain.[14] Low-level lasers at a wavelength of 650nm are also used in the treatment of oral-facial pain, such as mucositis pain.[22] Therefore, this study employed a 650nm laser diode light.

In conclusion, the results for 650nm laser diode light showed that 35-second irradiation produced higher scores for viability and proliferation caused by the energy absorbed by the cells which is sufficient to stimulate their biological activity. Laser light exposed to hPDLF cells can trigger absorption of laser-emitted photons by the cell chromophore. Conversely, a biostimulation response ensues due to accelerated electron transport reactions which can, in turn, cause an increase in ATP production. Increasing ATP synthesis in mitochondria then accelerates the speed of cell mitosis. The effects of laser biostimulation can increase the secretion of growth factors, such as TGF-β, which are responsible for inducing collagen synthesis from fibroblasts. TGF-β is involved in cell proliferation, which can extend the lifespan of fibroblast cells.[10] In conclusion, irradiation using a 650nm laser diode with irradiation times of 15 seconds and 35 seconds can cause an increase in hPDLF cell viability and proliferation.

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