Low-level laser therapy parameters to promote wound healing with diode laser

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Figures: 4

Tables: 0

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Abbreviations: Low-level laser therapy (LLLT), human gingival fibroblast (hGF), Watts (W)

Abstract:
Lasers are widely used in dentistry and offer an array of clinical applications, such as "photobiomodulation," which utilizes low-level laser therapy (LLLT) to achieve stimulatory effects within tissue. The purpose of this *in vitro* study is to determine the optimal power (Watts) setting and duration of irradiation using a diode laser, and its effect on human gingival fibroblasts (hGF). In this study, plated hGF were irradiated at 2W, 4W, 6W, 8W, and 10W for either 1- or 2-minutes and cell survival was assessed. For the second part of this study a scrape was made within each well of the plated hGF, and then LLLT was performed for 1 minute at 0W, 2W, and 4W and examined for three days. Results from the cell survival assay revealed approximately 100% survival for all power outputs (0-10W) in the 1-minute treatment groups and also in the 2-minute treatment groups at 0W, 2W, and 4W; however, in the 2-minute group survival began to significantly decrease at 6W and higher. Regarding the wound contraction assay, there was significantly more wound closure at both 2W and 4W compared to the control at days 1 and 2. Furthermore, the 2W group demonstrated statistically more wound closure at day 1 compared to 4W treatment group. By day 3, no differences were observed between any of the groups. The findings from this study found LLLT with a diode to have a stimulatory effect on hGF and promote faster wound closure, the laser parameters which had the most stimulatory effect *in vitro* was 2W and 1 minute of irradiation.

Introduction:

Lasers are widely used in dentistry and have various applications, such as, frenectomies, gingivectomies, pulpotomies, sulcular debridement, teeth whitening, etc. Another application of lasers in dentistry is "photobiomodulation," which utilizes low-level laser therapy (LLLT). By definition, LLLT uses an energy density of <100 J/cm^2 to achieve its stimulatory effects within tissue, and also releases minimal heat as a byproduct, thereby preventing tissue injury (Chung, 2012). The biostimulatory properties of lasers have been shown to accelerate healing (Dawood, 2013) (Hopkins, 2004) and reduce pain (Lim, 1995) (Bjordal, 2003) through its tissue penetrating wavelengths and ability to promote molecular activity. LLLT was first utilized in the medical field, prior to its introduction to dentistry (Chung, 2012). Consequently, extra-oral wound healing has been focused on more in the literature (Hopkins, 2004) (Hussein, 2011),

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with less attention directed towards intra-oral wound healing (Choung, 2019). Nonetheless, numerous histological and animal studies have evaluated the effect of LLLT on gingival fibroblasts, intra-oral wound contraction, cytokine expression, growth factor secretion, etc. The literature on LLLT has demonstrated its ability to increase gingival fibroblast proliferation, increase expression of Col-1 and growth factors (hEGF, hKGF, IGF) (Hakki and Bozkurt, 2012), decrease MMP-2 and MMP-9 secretion (Da Silva, 2013), inhibition of proinflammatory cytokines (TNF-α, IL-6, and IL-8) (Basso, 2015) and enhanced intra-oral wound closure (Wang, 2015) (Walsh, 1997). These results are promising to the field of periodontology, specifically, as post-surgical wound healing is pertinent to every day practice.

While *in vitro* and animal studies have demonstrated enhanced wound healing with LLLT, the superiority of LLLT is less apparent in human clinical studies (Choung, 2019). One proposed explanation for the lack of clinical impact may be a reflection of the lack of consensus on parameters (Walsh, 1997) and the delicate dose-dependent relationship exhibited by the laser settings (Karu, 2010) (Illescas-Montes, 2017). Proper dosing of photobiomodulation is critical in order to obtain stimulatory effects, however it is often difficult to determine optimal dosage (Convissar, 2011). In fact, according to Arndt-Schlutz law, dosages of LLLT must fall within a certain therapeutic window in order to promote biostimulatory effects, and doses exceeding that range actually inhibit these properties (Convissar, 2011).

Diode lasers (655-980nm) have gained recent popularity due to their lower cost, smaller size, and ability to penetrate tissues (Choung, 2019). A recent systematic review on diode lasers found the energy density range for stimulatory effects on human gingival fibroblasts to be 0.5-16 J/cm^2 and at 2-10 J/cm^2 for human PDL fibroblasts (Ren 2016). A more narrow range was observed in a study by Illescas-Montes, R. et al. (2017) which reported stimulatory effects on human fibroblast as long as the power of the diode laser did not exceed 0.5 W and the energy density was between 1-4 J/cm^2. In contrast, studies by Harorli (2019) and Hakki and Bozkurt(2012) failed to show increased cell proliferation and viability of gingival fibroblasts at 0.3W, 0.5W, 0.7W, 1.5W or 2W.

The purpose of this *in vitro* study is to determine the optimal power (W) settings and duration of irradiation using a diode laser by testing its effect on cell viability and wound contraction of human gingival fibroblasts (HGF). This study will provide further insight into the parameters required for LLLT to promote intra-oral wound healing.

Methods:

This *in vitro* study consisted of three experiments to test the effect of LLLT on human gingival fibroblasts (hGF) at varying parameters using the Biolase EPIC X™ (InGaAsP diode laser). A brief overview of the study is as follows. In the first part of the study, a survival assay was performed to test the effect of exposure times. The hGF were treated at a power setting of 10W for 0, 1, and 2 minutes. Following treatment, the cells were incubated for 24 hours, dyed with calcein, and then survival was assessed using a plate reader. A similar survival assay was conducted again in the second part of this study, but instead explored the survival of hGF testing six different power settings (0W, 2W, 4W, 6W, 8W, 10W) at exposure times of 1 minute and 2 minutes. The third and final part of this study was a cell motility assay to assess hGF migration at three different power settings (0W, 2W,

and 4W) at constant exposure time of 1 minute. Cell motility of the treated cells was examined at Days 0,1,2, and 3.

Laser Parameters:
Biolase EPIC X™ (InGaAsP diode) using "Deep tissue handpiece"
Wavelength: 940nm
Deep tissue HP tip area= 7.1cm^2, 30mm diameter
Non-contact mode, distance of laser to plate= 1mm
Transmission mode: CW
Output power: 0W, 2W, 4W, 6W, 8W, 10W

Cells: Gingival fibroblasts (GF) established from a patient with healthy gingiva who underwent oral surgery at the Louisiana State University School of Dentistry, will be used for this study. Tissues were obtained following informed consent as approved by the Institutional Review Board. The donor was a 13 year old boy with no systemic diseases who underwent periodontal surgery (Palaiologou et al., 2001). As described previously (Lallier et al., 2005) cells will be maintained in minimum essential medium *alpha* (MEMα) containing 10% fetal calf serum (FCS) and 200 units/ml of penicillin and 200 μg/ml streptomycin (GIBCO Grand Island, NY). Gingival fibroblasts between the 12th and 18th passage are deemed to be "rapidly dividing", while cells after the 33rd passage are deemed to be "senescent", for this study. After passage 30 these cells tend to slow their rate of proliferation, eventually becoming non-mitotic around passage 33.

Cell Survival Assay: 20,000 cells are added to the 24-well plates proteins in a volume of 400 μl of MEM containing 10% fetal bovine serum. Cells are allowed to adhere to the substratum 24 hours. Four samples are prepared for each extracellular matrix (ECM) protein for each experiment. Non-adherent cells are removed by three washes of 500 μl of PBS. The adherent cells are quantified fluorometrically using Calcein-AM fluorescent dye (Molecular Probes, Eugene, OR). This dye is a non-fluorescent esterase substrate that is readily taken into live cells. Once internalized, this dye is cleaved by endogenous esterases and rendered fluorescent. Therefore, this dye readily labels the cytoplasm of live cells. The sample fluorescence is measured using a fluorescence microplate reader (Bio – Tek Instruments FL600, Winooski, VT) with filters appropriate for ~ 480nm excitation and ~520nm emission. All data will be collected and each group of eight samples will be averaged and a mean and standard deviation will be compared to the control value according to the following formula.

Cell Motility Assay: In order to examine cell motility, cells were plated into 24-well tissue culture plates, and allowed to adhere for 24-hours. Using a P-1000 pipette tip, a 1.5 mm wound was made in each well. Cells were immediately treated with either 0W, 2W, or 4W for 1 minute, and the width of the wound was measured at 24-hour intervals. Untreated cells served as controls. For each time point and treatment, 25 linear measurements were made to measure the distance between cells on both sides of the scrape. Cells were treated with Calcein AM for 1 hour to aid in visualization. Cells were examined using a Nikon inverted TE2000 microscope equipped with a CoolSNAP cfi camera and Metamorph software. For quantitative analysis, 25 measurements were averaged. The mean and standard deviation were compared to the control values for untreated cells.

Statistical Analysis: For quantitative analysis, eight samples were averaged and the mean and standard deviation were compared to the control values for untreated cells. Comparisons of experimental groups were performed via ANOVA where normality of the data was confirmed using the Shapiro-Wilk normality test, with $P < 0.05$ considered to be significant.

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‡ Invitrogen, GIBCO, Grand Island, NY
††† Invitrogen, Molecular Probes, Eugene, OR, USA
‡‡‡ Zeiss Primovert microscope, Germany
§ AmScope Mu1203-FL digital camera, Irvine, CA, USA
* ImageJ, NIH, Bethesda, MD, USA
†††† BioTek Synergy2, Winooski, VT, USA

Results:

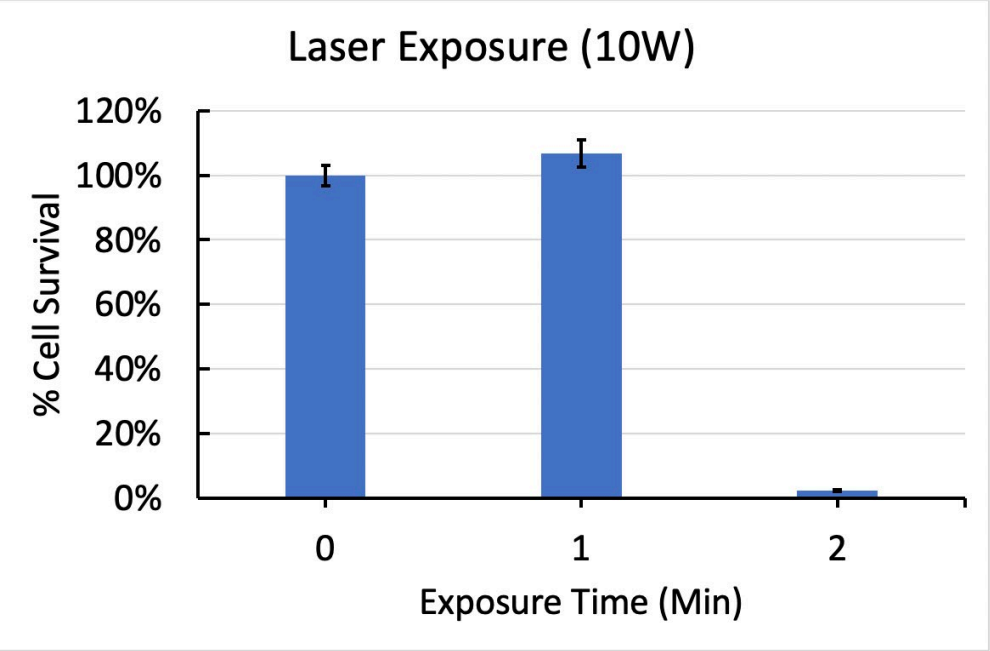

Figure 1- Shows the percent hGF cell survival rate after LLLT at 10W for either 0, 1, or 2 minutes duration. Results show approximately 100% survival for both control and 1-minute treatment groups, whereas the 2-minute treatment group yielded approximately 0% survival.

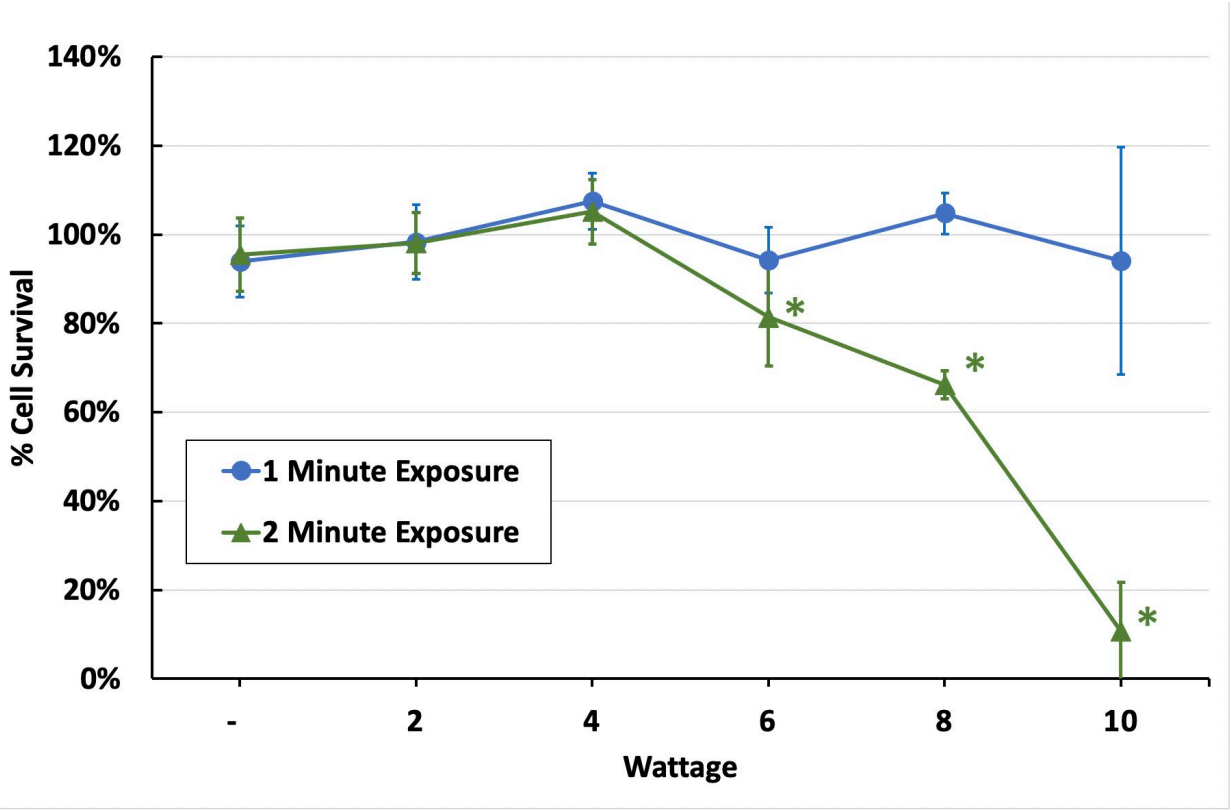

Figure 2- Shows the percent hGF cell survival rate after LLLT at varying powers for either 1- or 2-minute duration. An asterisk (*) denotes a statistically significant difference ($p<0.05$) between the two treatment groups. Results show approximately 100% survival for all power outputs (0-10W) for the 1-minute treatment groups. In comparison, the 2-minute treatment groups demonstrated 100% hGF survival at 0W, 2W, and 4W, however survival began to significantly decrease at 6W and above, approaching almost 0% survival at 10W.

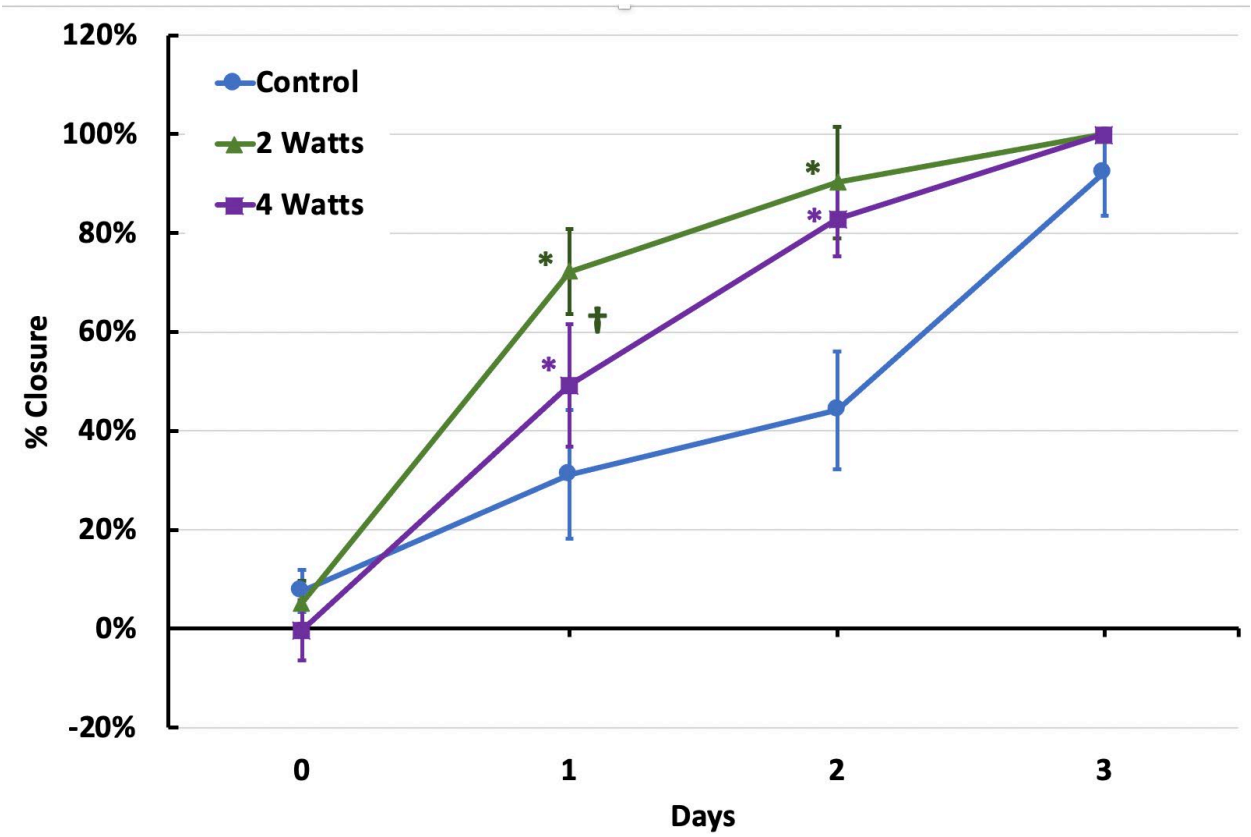

Figure 3- Shows the percent closure over of the scrape assay over 3 days. On day 0 hGF were treated with either 0W (control), 2W, or 4W for 1-minute duration, and the width of the wound was measured at 24-hour intervals. An asterisk (*) denotes a statistically significant difference between the treatment group and the control and a (†) denotes a statistical difference between the two treatment groups. An advantage of both 2W and 4W is seen over the control at days 1 and 2, whereas a 2W demonstrates a statistically more wound closure at day 1 compared to both the control and 4W treatment groups.

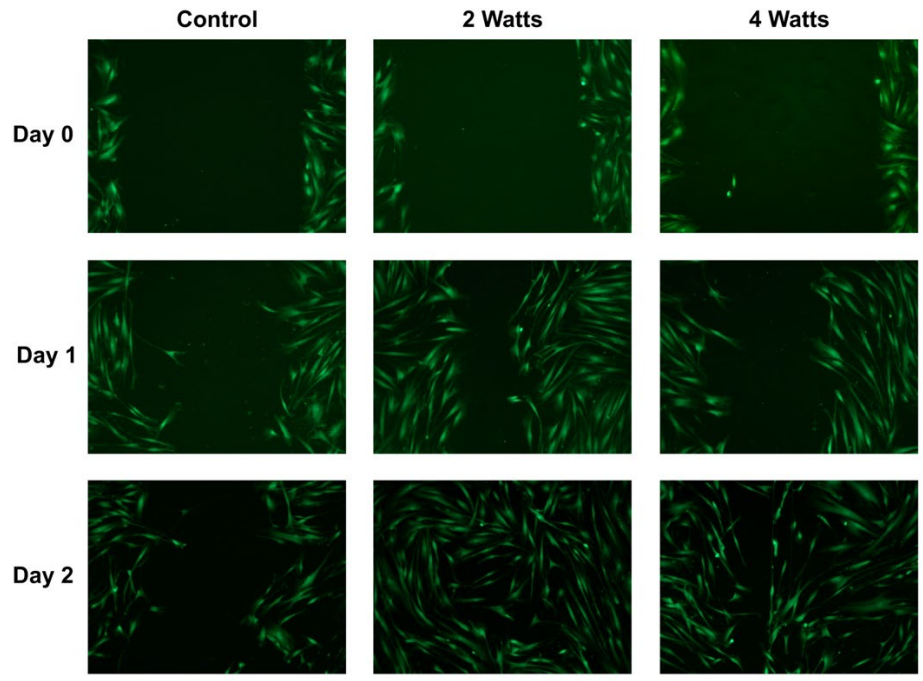

Figure 4- This figure contains photos taken from the scrape from which the data in Figure 3 was derived. The hGFs were dyed with Calcein AM and photos were captured at days 0, 1, and 2 with the CoolSNAP cfi camera and Metamorph software. These photos illustrate a wound inflicted in all groups at day 0. By day 1, the 2W treatment group demonstrates significantly more wound closure than the control and 4W. By day 2, both 2W and 4W groups demonstrate full wound closure, while only partial closure is observed in the control group.

LLLT- Exposure Duration and Output Power

Human gingival fibroblast survival was affected by the duration of laser exposure, particularly when longer exposure times were performed at higher power settings. The first experiment demonstrated that both the control (no exposure) and a 1-minute treatment were conducive to fibroblast survival at a power level of 10W. However, cell death was observed when the exposure time was increased to 2 minutes at 10W, suggesting these parameters were outside of the therapeutic window of LLLT and harmful to the cells.

A similar trend for exposure time was observed in the second experiment. A 1-minute treatment duration promoted approximately 100% GF survival at all power levels (2W, 4W, 6W, 8W, and 10W). One hundred percent cell survival was also observed when exposure duration was increased to 2-minutes, but only at 2W and 4W power settings. When the 2-minute laser treatment was increased to a power of 6W, there was a significant decrease in cell survival (81%). This trend continued in a dose-dependent manner, with even greater decreases in cell survival at 8W and 10W (66% and 11%, respectively). This data suggested there was no negative impact on cell survival using LLLT at 1-minute duration or with lower power settings for 2 minutes.

The results of the scrape assay revealed differences in motility of HGFs at varying output powers. The scrape assay tested power levels of 0W, 2W, and 4W at 1-minute of exposure. The 1-minute duration time was chosen based off the beneficial results obtained in the cell survival assay. An advantage of both 2W and 4W was seen over the control at day 1, with the control group demonstrating 31% closure of

the original wound, while the 2W group demonstrated 72% closure, and 49% closure was observed in the 4W group. Statistically significant differences were observed for both 2W and 4W over the control groups at days 1 and 2, favoring greater wound closure for the treatment groups. Furthermore, the 2W group demonstrated significantly greater % wound closure than the 4W group on day 1. By day 2, both treatment groups demonstrated superior wound closure to the control (90% [2W], 83% [4W], and 44%[control]). These differences were no longer observed by day 3, with 92% closure observed in the control groups and 100% closure for both treatment groups.

Discussion:
The findings from this study show that hGF survival and motility are affected by the duration of exposure to LLLT and the power output. The results of this *in vitro* study suggest 1-minute of exposure is superior to 2-minutes in terms of cell survival, particularly when higher power outputs are used (6W and above). Perhaps of more clinical relevance, results from the cell motility assay suggest LLLT promoted faster wound closure than the control during the early healing phase (days 1 and 2). Furthermore, using a power setting of 2W for 1 minute resulted in superior wound closure on day 1 compared to 4W, providing further insight for the appropriate laser parameters when utilizing LLLT.

In agreement with the findings from this study, a recent systematic review on LLLT with diode lasers concluded that LLLT stimulated human gingival fibroblasts *in vitro* with regards to cell viability, proliferation, migration, and protein synthesis (Bakshi, 2022). They found it was possible to establish a suitable power setting for a particular wavelength spectrum, but no other parameters could be established due to the lack of reporting within the literature. The systematic review concluded that for diode lasers in the 800-900 nm range, LLLT promoted cell growth when the power setting was 10mW, whereas adverse outcomes were observed when the power was ≥0.5W (Bakshi, 2022). While those suggested power levels are much lower than the 2W used in this study, it is important to note the diode used in this study has a wavelength of 940nm and is therefore out of the aforementioned range. The systematic review could not determine the therapeutic range for power level (W) using diodes with wavelengths >900nm because there was only one study available (Bakshi, 2022) and thus a lack of literature.

The benefits of LLLT using a diode to improve wound healing was also observed in a randomized clinical split-mouth study by Kohale (2018). The study showed that three applications of LLLT on day 1, 3, and 7 following a gingivectomy significantly accelerated re-epithelialization and healing of the wound compared to sites that did not receive LLLT (Kohale, 2018). As expected, there were differences in the parameters used for Kohale's (2018) *in vivo* study, compared to this *in vitro* study. Kohale (2018) irradiated the surgical site for 40 seconds, which was similar to exposure time used in this study. However, in contrast to the 2W of power used in our experiment, Kohale (2018) used a much lower power setting of 100 mW for LLLT application. This discrepancy reflects one shortcoming of *in vitro* studies, which limits the ability to standardize laser parameters amongst *in vitro* and *in vivo* studies.

Conclusion: Low-level laser therapy using a diode laser promotes gingival fibroblast survival and accelerates fibroblast migration for faster wound closure during the early healing phase.

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