EFFECTS OF INFRARED LASER EXPOSURE IN A CELLULAR MODEL OF WOUND HEALING

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To investigate the efficacy of low-level exposure to 980 nm laser light, we have measured cell growth rates following wound induction using an in vitro model of wound healing. A small pipette was used to mechanically induce a wound in fibroblast cell cultures, which were then imaged at specific time intervals following wound induction and exposure to various doses of laser light. Our results show that exposure to low and medium intensity laser light significantly accelerate cell growth; high intensity laser light negated the beneficial effects of laser exposure on cell growth. Further experiments demonstrated that cell growth was accelerated over a wide range of exposure durations using medium intensity laser light with no marked reduction in cell growth at the longest exposure durations. Our results confirm clinical observations that low-level exposure to 980 nm laser light can accelerate healing of superficial wounds. However, these results also demonstrate the need for appropriate supervision of laser therapy sessions to prevent overexposure to laser light that may reverse increases in cell growth rates observed in response to lower levels of laser exposure.

Introduction

Photobiotherapy is the clinical application of light for healing decubitus ulcers (bedsores) and other superficial wounds. Previous studies have demonstrated significant clinical value for the use of low-level laser light to accelerate the healing of superficial wounds (Mester et al., 1985). Although the cellular mechanisms of this accelerated wound healing are not known, a recent study has demonstrated that low-level light from a 633 nm HeNe laser accelerates cell growth in a cellular model of wound healing and improves cellular metabolism in a dose-dependent manner (Hawkins and Abrahamse, 2006).

The use of infrared (IR) light may have significant advantages compared to visible light for clinical applications. In particular, the longer wavelength light minimizes scatter produced by superficial layers of the skin, and allows for a penetration of the light into deeper layers of skin that are most active during wound healing processes. In addition, IR light produces heating of deeper skin layers, promoting increased blood flow and to further accelerate healing processes (Dierickx, 2006).

Although IR light has demonstrated clinical value, the effects of IR light at the cellular level have not been examined. To determine whether IR can improve cell growth and recovery, we have utilized a clinical IR laser in a cellular model of wound healing. Our results show that limited doses of IR light can increase the rate of cell growth within hours of light exposure.

Methods

Cell culture

Fetal human skin fibroblast cells (cell line CCD-1070SK, American Type Culture Collection) were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 1 mM L-glutamine, 1% penicillin-streptomycin and 3% fetal bovine serum. The cultures were incubated at 37 ºC with 95% O_2 - 5% CO_2 at 85% humidity. Cells were trypsinized using a 0.25% (w/v) trypsin and 0.03% EDTA solution in DMEM and seeded into sterile 35 mm polystyrene culture dishes at a density of 7.0×10^4 cells per cm^2. Cells were incubated overnight to allow the cells to recover from trypsinization and to adhere to the bottom of the culture dishes.

Wound healing model

Culture dishes with plated cells were removed from the incubator, placed on the stage of an inverted microscope equipped with relief contrast optics (IX-71, Olympus) and visualized using a 4x, 0.13 numerical aperture objective. To simulate a wound, confluent monolayers were scratched with a sterile pipette approximately 1 mm in diameter (figure 1). Following wound induction, the output of a 7.5W, 980 nm laser used for clinical applications (VTR 75, Avicenna Laser Technologies) was focused on a spot 12.5 mm in diameter centered on the wound with the visible red aiming beam disabled. Cell growth into the wound region at time intervals up to 48 hours post-exposure was compared to control dishes in which no laser light was used. To attenuate the laser output and focus the light on a smaller spot, a 3 mm fiber approximately 1.5 m in length was coupled to the laser output and directed toward the center of the 35 mm culture dish approximately 10 mm above the dish surface. Two different sets of experiments were performed: the first compared different exposure intensities over the same exposure time, the second compared different exposure times at the same exposure intensity. For the first set of experiments, the laser output was varied from 1.5 - 7.5 W to produce exposure densities 26 - 120 mW/cm^2 for 2 minutes, resulting in exposure doses from 3.1 - 14.4 J/cm^2. For the second set of experiments, the laser output was fixed at 4.5 W or 73 mW/cm^2 and the exposure times were varied from 20 sec to 15 min, resulting in exposure doses from 1.5 - 66 J/cm^2.

Image acquisition and analysis

To assess the growth of cells back into the wound region, images were acquired at hourly intervals up to 8 hours after wound induction and laser exposure, and then at 24 and 48 hours post exposure. To maintain a controlled environment, culture dishes were returned to the incubator between image acquisition sessions. Images were acquired using a Quantix 57 scientific grade digital CCD camera (Roper Scientific) using custom made software designed to run under Matlab (Mathworks). To assess cell growth, an automated analysis routine was developed to measure the area of the imaged field that was covered by cells. This procedure utilized the Matlab edge detection function edge( ) with the Canny algorithm option to detect lines of pixels along cell edges. Once edges were detected, the Matlab function imclose( ) was utilized to fill in the gaps between edge lines using circular structuring elements approximately one cell width across (12 - 15 pixels). The result of this analysis was the detection of pixels in regions covered by cells where edges were in proximity, whereas regions such as the wound that had little or no cell coverage were left undetected. Cell coverage area was then quantified at each time interval following wound induction and laser exposure by adding the number of detected pixels and comparing this to the coverage area immediately following wound induction. For each laser exposure dose and time interval following wound induction, images were acquired from five different culture dishes to provide repeated data samples for statistical analysis.

Statistical analysis

To separate the effects of recovery time and laser exposure dose, a two-factor analysis of variance (ANOVA) was performed on the cell coverage area data obtained from images acquired at different post-exposure elapsed times and from different laser exposure doses. Without any laser exposure, a complete re-growth of fibroblast cells into the wound region will occur over the 48 hour period during which cells were imaged. Therefore, a statistically significant increase in cell coverage area will occur over the course of the experiment. However, the two-factor ANOVA procedure allows for the separation of two experiment factors, in this case elapsed time and laser exposure dose. Therefore, statistically significant effects of laser exposure dose can be compared to the effects of elapsed time, and can provide an estimate of the acceleration of cell growth by the various laser exposures. The statistical software package Minitab 14 (Minitab Inc.) was utilized to perform the two-factor ANOVA; data was entered as three columns with elapsed time, exposure dose and percent increase in cell coverage area from images immediately taken after wound induction.

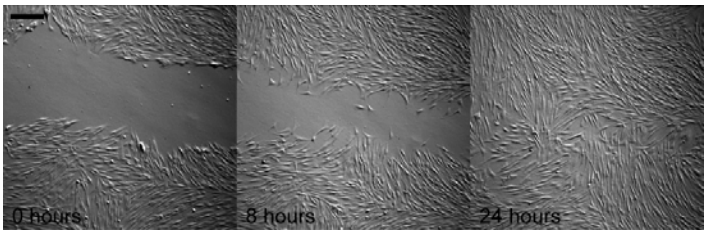

Figure 1: cell culture model of wound healing. A sterile pipette approximately 1 mm in diameter is used to induce a wound in a monolayer of fibroblast cells plated onto 35 mm culture dishes (far left image). Fibroblast cells grow back into the wound region within 8 hours (middle image) and the wound is completely overgrown within 24 hours. Scale bar: 250 μm. The algorithm used to calculate cell coverage area found that 64.9%, 76.7% and 97.7% of the imaged area was covered with cells in the three images displayed above.

Results

Our results demonstrate that exposure to light from a 980 nm laser can enhance cell growth rates in an in vitro wound model. A range of exposure doses was investigated by varying laser output power over a fixed exposure duration, or by varying exposure duration at a fixed laser output power. Figure 2 shows the results of the first experiment in which laser output power was varied from 1.5 - 7.5 W to produce an exposure level of 26 - 120 mW/cm^2 over a two minute exposure, resulting in exposure doses from 3.1 - 14.4 J/cm^2. Regardless of exposure level, significant cell recovery was observed within three hours of wound induction; however, exposure to moderate levels of laser light (26 - 97 mW/cm^2) appeared to enhance cell growth at all time intervals relative to control experiments in which no laser exposure was applied (figure 2, top panel). These results were confirmed by the results of a two-factor ANOVA (figure 2, lower right), which shows that significant increases in cell growth were observed with two minute exposures to 26 - 73 mW/cm^2 ($p < 0.01$) and 97 mW/cm^2 ($p < 0.05$). These results also show that the beneficial effects of laser exposure are negated by over-exposure: fibroblasts exposed to 120 mW/cm^2 of laser light for two minutes did not show any significant increase in growth rates relative to control experiments.

The two-factor ANOVA analysis also provided a measure of how much cell growth was accelerated by laser exposure. Over the first eight hours following wound induction, the average cell coverage increased linearly by approximately 1.7% per hour (figure 2, lower left); the growth rate begins to slow before 24 hours, when cells across the wound margin begin to contact each other and completely fill the area previously devoid of cells. When compared to the mean cell growth measured at various

time intervals following wound induction, the 4 - 6% increase in cell growth produced by 49 - 73 mW/cm^2 of laser exposure over a two minute period represented an acceleration of wound healing by approximately 2.5 - 3.5 hours within the first eight hours of healing. This represents a sizeable acceleration in cell growth considering that the wounds from our in vitro model were nearly completed healed within 24 hours following wound induction. Despite the significant increases in cell growth across various time intervals and exposure levels, the two-factor ANOVA analysis did not find any significant interaction between elapsed time and exposure level. In other words, the various exposure levels showed consistent effects across all time intervals following wound induction, and there were no exposure levels whose effects were only observed at a particular time interval or subset of time intervals following wound induction.

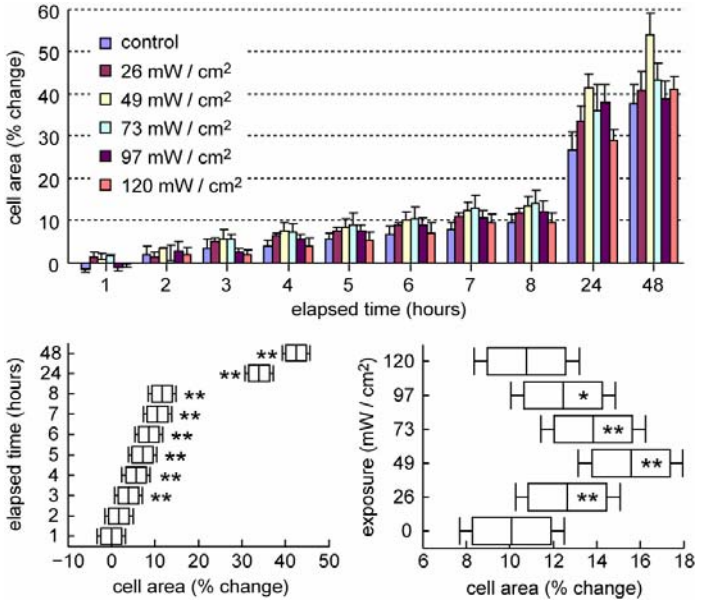

Figure 2: *Top panel:* cell growth in wound model as a function of time elapsed from wound induction and laser exposure intensity. Vertical bars show % change in cell coverage area averaged across five experiments in which cells were not exposed to laser light, or in which cells were exposed to 26 - 120 mW/cm^2 of light during a two minute exposure by varying the laser power from 1.5 - 7.5 W to give exposure doses from 3.1 - 14.4 J/cm^2. Error bars show S.E.M across five experiments. *Bottom left:* confidence intervals from the two-factor ANOVA analysis demonstrate a significant increase in cell coverage area observed as early as 3 hours after wound induction regardless of laser exposure (** $p < 0.01$). Horizontal bars show 95% confidence intervals with midline at mean value; error bars show 99% confidence intervals. *Bottom right:* confidence intervals from the two-factor ANOVA analysis demonstrate a significant increase in cell coverage area for low and moderate levels of laser exposure when compared to no laser exposure (** $p < 0.01$ for 26 - 73 mW/cm^2; * $p < 0.05$ for 97 mW/cm^2). No significant increase in cell coverage was observed at the highest exposure level (120 mW/cm^2).

Figure 3 shows the results of the second experiment in which exposure durations were varied from 20 sec - 15 min at a constant laser output power of 4.5 W to produce an exposure level of 73 mW/cm^2, resulting in exposure doses from 1.5 - 66 J/cm^2. As with changes in exposure level, significant cell recovery was observed within three hours of wound induction regardless of exposure duration, and a wide range of exposure durations appeared to enhance cell growth at all time intervals relative to control experiments in which no laser exposure was applied (figure 3, top panel). These results were confirmed by the results of a two-factor ANOVA (figure 3, lower right), which shows that significant increases in cell growth were observed with 73 mW/cm^2 exposures having durations of 20 sec - 2 min to produce exposure doses of 1.5 - 8.8 J/cm^2 ($p < 0.01$). Note that a long exposure of 15 min (65.7 J/cm^2) produced a significant decrease in cell growth ($p < 0.05$), suggesting that long exposure to laser light at a moderate level can reverse the benefits of lower exposure does. A comparison of these results to the mean cell growth measured at various time intervals following wound induction (figure 3, lower left) showed that the 6% increase in cell growth produced by 73 mW/cm^2 of laser exposure over a 20 sec - 2 minute period represented an acceleration of wound healing by approximately two hours within the first eight hours of healing. Furthermore, the two-factor ANOVA analysis did not find any significant interaction between elapsed time and exposure duration despite the significant increases in cell growth across various time intervals and various exposure durations.

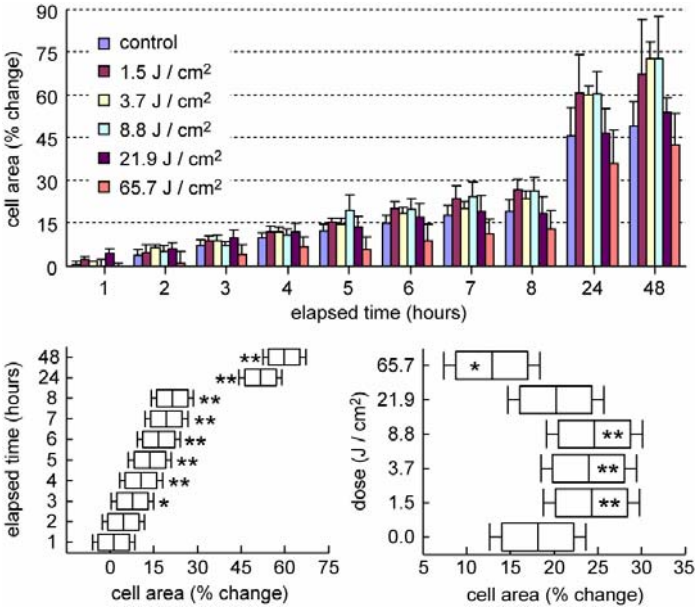

Figure 3: *Top panel:* cell growth in wound model as a function of time elapsed from wound induction and laser exposure dose. Vertical bars show % change in cell coverage area averaged across five experiments in which cells were not exposed to laser light, or in which cells were exposed to 73 mW/cm^2 of light during exposures that varied from 20 sec to 15 min to give exposure doses from 1.5 - 65.7 J/cm^2. Error bars show S.E.M across five experiments. *Bottom left:* confidence intervals from the two-factor ANOVA analysis demonstrate a significant increase in cell coverage area observed as early as 3 hours after wound induction regardless of laser exposure (* $p < 0.05$ for 3 hours, ** $p < 0.01$ for 4 hours and beyond). *Bottom right:* confidence intervals from the two-factor ANOVA analysis demonstrate a significant increase in cell coverage area for moderate laser exposure doses when compared to no laser exposure (** $p < 0.01$ for 1.5 - 8.8 J/cm^2). No significant increase in cell coverage was observed at the second highest dose (21.9 J/cm^2), and a significant decrease in cell coverage was observed at the highest exposure dose (* $p < 0.05$ for 65.7 J/cm^2).

Conclusions

Our results confirm the clinical observation that low-level exposure to 980 nm of laser light can accelerate cell growth in a wound healing model. Because our measurements were obtained from an in vitro cell culture model, these results also suggest that the mechanisms involved in the acceleration of cell growth following laser exposure are cellular or molecular in nature. The hypothesis that IR light accelerates healing processes by heating skin and promoting increased blood flow (Dierickx, 2006) could not explain the increased cell growth rates in an in vitro cell culture model. Our measurements suggested that IR exposure produced temperature increases less than 2 ºC, and the use of a controlled incubation environment between image acquisition intervals further minimizes the temperature variability in our experiments. Previous researchers have suggested that light exposure increases ATP levels by altering the energetic state of light-sensitive cytochromes within the inner mitochondrial membrane that participate in oxidative phosphorylation (Karu et al., 1995); we are conducting further experiments to examine ATP levels following low-level exposure to 980 nm laser light.

Our results also demonstrate the importance of appropriate supervision of laser light exposure in a clinical setting. In particular, the average cell growth rates formed a non-monotonic function of laser exposure levels (figure 2, lower right) and exposure doses (figure 3, lower right); with peak growth rates at moderate exposures, and reduced benefit at higher exposure intensities and doses. This suggests that excessive light exposure could have potentially damaging effects that negate any initial benefit of light exposure. Therefore the appropriate exposure levels and durations must be selected in order to maximize cell growth rates.

References

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