Importance of pulsing illumination parameters in low-level-light therapy

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Abstract. The influence of emission parameters in low-level-light therapy on cellular responses is not yet fully understood. This study assessed the impact of various light delivery modes on collagen production in human primary fibroblast cultured in monolayers after three treatments with red light-emitting diode illumination (630 nm, 8 J/cm^2). Human type I collagen was measured in cell culture supernatants with procollagen type I C-peptide enzyme immunoassay. Results demonstrated that, 72 h post-baseline, specific microsecond pulsing patterns had a more favorable impact on the ability of fibroblasts to produce collagen *de novo* than comparative conditions of continuous wave, pulsed 50% duty cycle, and millisecond pulsing domains. The cascade of events leading to collagen production by red illumination may be explained by the photodissociation of nitric oxide from cytochrome *c* oxidase. Short and intermittent light delivery might enhance this cellular event. © *2010 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.3477186]

1 Introduction

The use of low-level-light therapy (LLLT) in an ever-growing number of clinical indications is constantly evolving. The large number of illumination parameters now available adds to the medical applicability but also to its complexity. It has been suggested that LLLT can modulate skin cellular processes as a result of light-emitted photon absorption by mitochondrial chromophores.[1] The influence of emission parameters on cellular responses is not yet entirely understood.

Both pulsed and continuous wave (CW) modes are available in LLLT devices, which provide medical practioners with a wide range of therapeutic options. The relative influence of CW and pulsed modes on cellular response has not been fully studied. Until now, comparative studies have shown conflicting results.[2–7] For instance, no difference between these delivery modes was reported in an experiment by Al-Watban and Zhang.[5] Results from this study revealed that the frequency of a pulsed laser was not found to increase wound healing in rats compared with a CW laser (635 nm, 0.89 mW/cm^2, 1.0 J/cm^2). Experience in our laboratory using a light-emitting diode (LED) yielded opposite results. In our hands, sequentially pulsed optical energy was more efficacious than the CW mode in stimulating collagen production in a suction blister model (660 nm, 60 mW/cm^2, 5 J/cm^2). Other studies have also obtained a similar pattern of results.[6] In a recent study by Brondon et al.,[7] photoradiation outcomes following delivery of 670-nm (10-mW/cm^2, 5-J/cm^2) light through a

0.025% melanin filter via continuous illumination or pulsed delivery at variable frequencies was examined. Pulsing had a significantly greater stimulatory effect on cell proliferation and oxidative burst as compared to the continuous photoradiation group. On the whole, the comparative efficacy of pulsed and CW modes on cellular processes remains an open question.

In addition, until now, limited attention has been dedicated to the understanding of the impact of pulsing structure on biological events. There is evidence in the literature that some biological processes are dependent on specific pulsing parameters. Pulsing frequency (pulse repetition rate and pulse duration) appears to hold such differential effects, as suggested by studies using different *in vitro* models.[7–10] In this regard, for instance, a wound healing study by El Sayed and Dyson[9] concluded that the observed increase in mast cell number was not pulsing frequency dependent, whereas degranulation was (820 ± 5 nm, 800 mW/cm^2, 21.6 J/cm^2). Moreover, past studies have shown that certain pulsing frequencies appear to be more efficacious than others in triggering desired biological outcomes.[7,10] Taken together, these findings underline the importance of studying pulsing parameters to achieve maximal stimulation of targeted cellular photobiomodulatory effects.

In sum, at this point in time, there is no clear picture of the relative impact of light delivery variables on specific molecular processes (e.g., collagen synthesis, cell proliferation, etc.). Moreover, it is unclear whether photobiomodulatory effects are similar across different cell lines, species, and patient types. Furthermore, damaged and "stressed" cells appeared to

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Fig. 1 Illumination device during photoactivation of fibroblasts cells in the 24-wells plate.

respond better to LLLT than normal cells.[11] In addition, past work has demonstrated that particular combinations of factors (e.g., wavelength, duty cycle) are necessary to achieve optimal results in specific models.[12,13] Finally, coherence of the delivered energy (photons) might also influence the outcome. All these elements may explain, at least in part, the discrepancy in results obtained until now with LLLT. Thus, better characterization of the influence of LLLT emission parameters on biological events is necessary. As a first step in this endeavour, one should assess, for a specific biological process, the relative influence of each parameter while controlling, as much as possible, for potential confounding variables. This was the aim of this study.

In the current experiment, we investigated the influence of various delivery modes using LEDs in the visible spectrum (630 nm, 8 J/cm^2) on type 1 procollagen stimulation in a model of human primary fibroblasts cultured in monolayers. The primary objective of this study was to identify, within a pulsing structure, the optimal parameters to enhance collagen production. More specifically, for each pulsing parameter examined (pulse duration, pulse interval, pulse train interval, and pulses per train), one level of each parameter was modified at a time to assess its impact on collagen stimulation. As a secondary objective, this study aimed at contrasting these pulsing structures to CW, millisecond pulsing domain, as well as regular pulsed 50% duty cycle conditions. Here, we report type 1 procollagen measurements performed 72 h postbaseline after three LED treatments. Results from this study should enable the identification of the optimal parameters to be used in LED protocols to enhance collagen production.

2 Methodology

2.1 Cell Culture and Irradiation Procedure

Human normal foreskin fibroblasts (CCD 1112Sk, CRL-2429 from ATCC) were cultured at 37 °C in 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS), and used between the fifth and seventh cellular passage. 70,000 cells/well were plated in a 24-well culture plate (equiglass cell culture plate, Genetix) (Fig. 1).

Table 1 Experimental conditions.

| Conditions	Parameter	Parameter Levels						
		Pulse Duration (μs)	Pulse Interval (μs)	Pulse Train Interval (μs)	Pulses per Train	Total Fluence (J/cm^2)	Exposure time (s)	Duty Cycle (%)
Microsecond pulsing	Reference	500	150	1,550	4	4.00	1000	50.0
	Pulse duration	100	150	1,550	4	1.33	1000	16.7
	Pulse interval	500	250	1,550	4	3.72	1000	46.5
		500	1000	1,550	4	2.44	1000	30.5
	Pulse train interval	500	150	750	4	5.00	1000	62.5
		500	150	50,000	4	0.31	1000	3.6
		500	150	100,000	4	0.16	1000	2.0
	Pulses per train	500	150	1,550	2	2.96	1000	37.0
		500	150	1,500	100	6.02	1000	75.3
Comparators	CW	100,000	0	0	1	4.00	500	100
	Millisecond pulsing	250,000	0	100,000	1	0.20	35	71.4
	Pulsed 50% duty cycle	250	0	250	1	4.00	1000	50.0
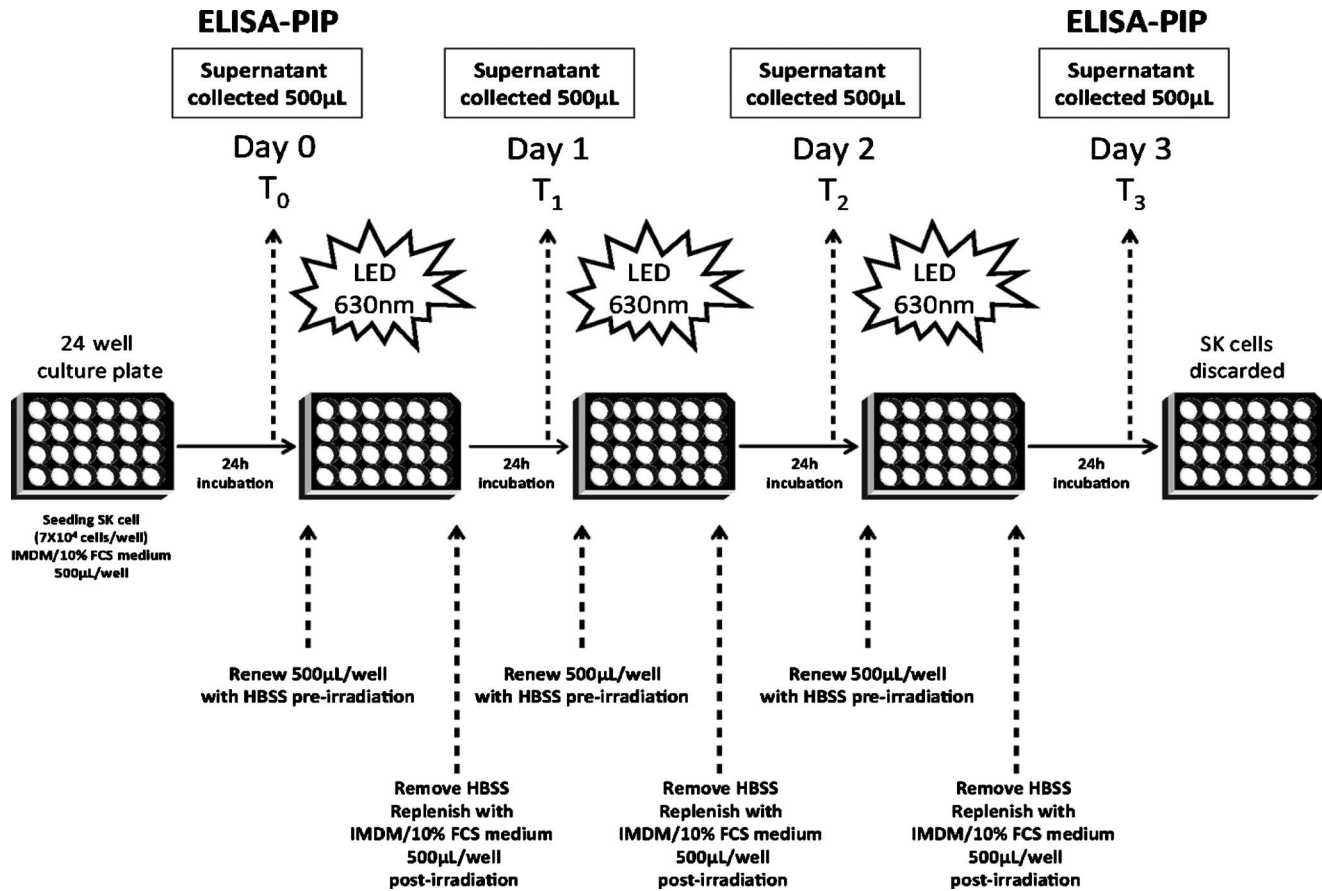
Fig. 2 Step by step procedure.

After 24 h and immediately before irradiation (T_0), supernatants were collected, and 500 μl of Hank's buffered salt solution (HBSS) was added. Cells were then irradiated according to specific delivery modes, as shown in Table 1, or exposed to a sham light source. Immediately following irradiation, HBSS was removed and replaced by 500 μl of cell culture medium (IMDM/10% FCS). Cells were next incubated at 37 °C for 24 h. This procedure was repeated three times at 24-h intervals. Supernatants were collected preirradiation at T_0, and 24 h postirradiation at T_1, T_2, and T_3. Supernatants were stored at −20 °C until procollagen type I
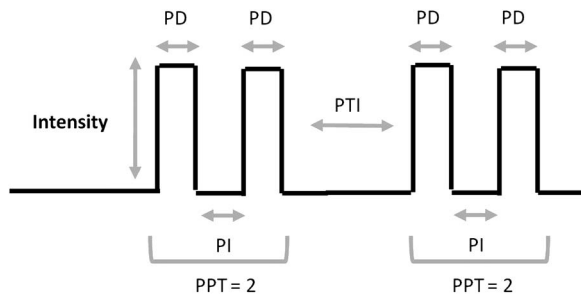

Fig. 3 Schematic representation of pulsing parameters: PD, pulse duration; PI, pulse interval; PTI, pulse train interval; and PPT, number of pulse per train.
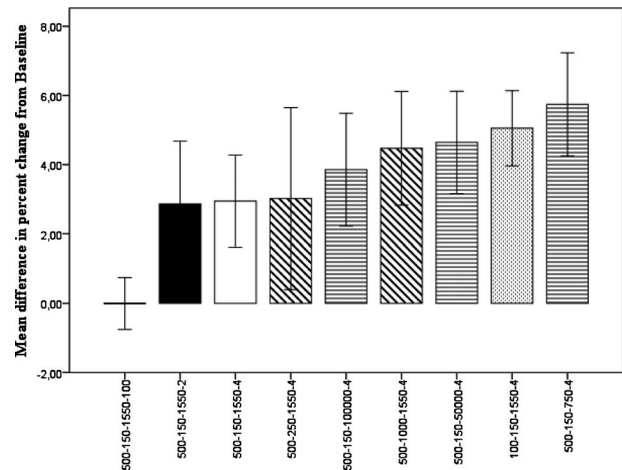

Fig. 4 Procollagen levels for the microsecond pulsing conditions (PD-PI-PTI-PPT). Data represented are mean difference in percent change from baseline ±2 standard error (SE): white, reference condition; dots, PD condition; oblique stripes, PI conditions; horizontal stripes, PTI conditions; and black, PPTI conditions.
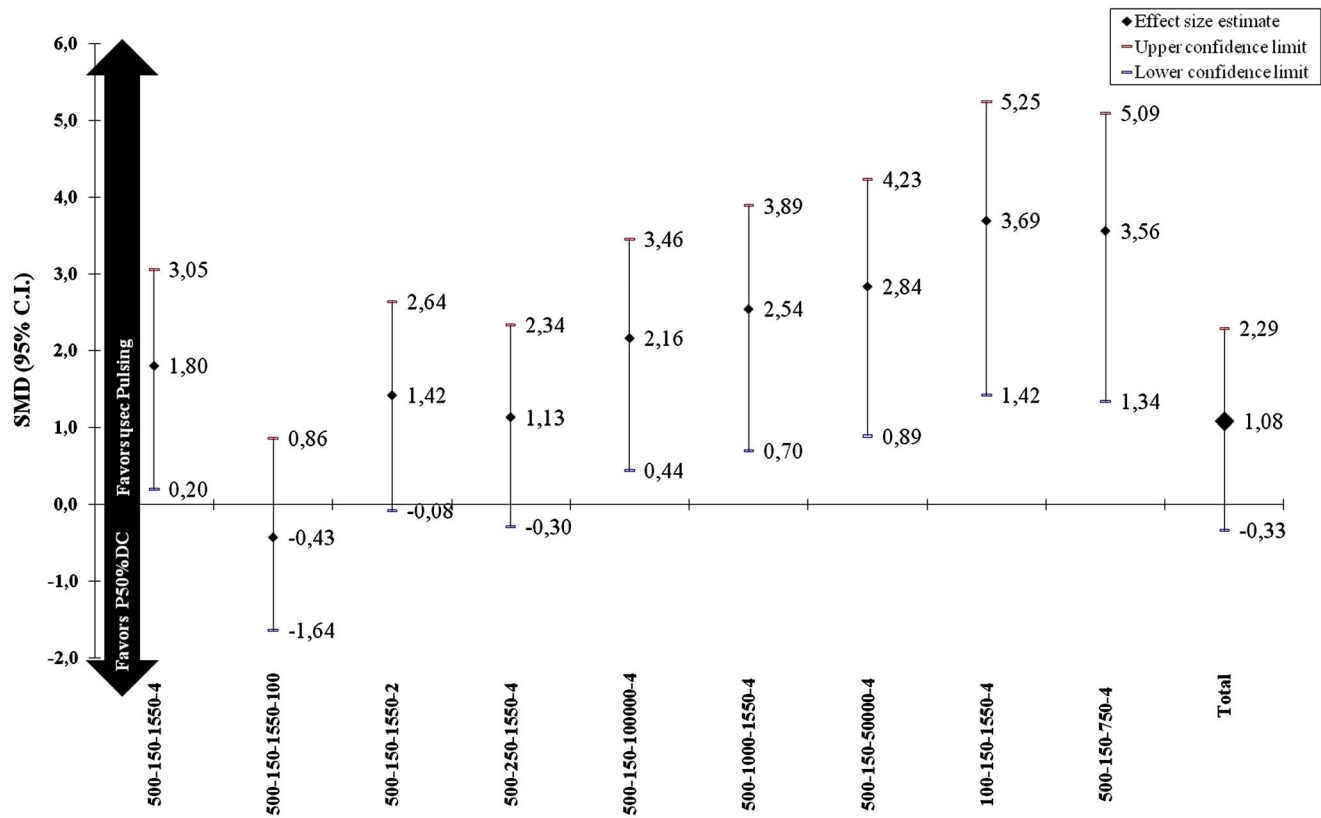
Fig. 5 Standard mean differences between microsecond pulsing and regular 50% duty cycle conditions. C.I.: confidence interval.
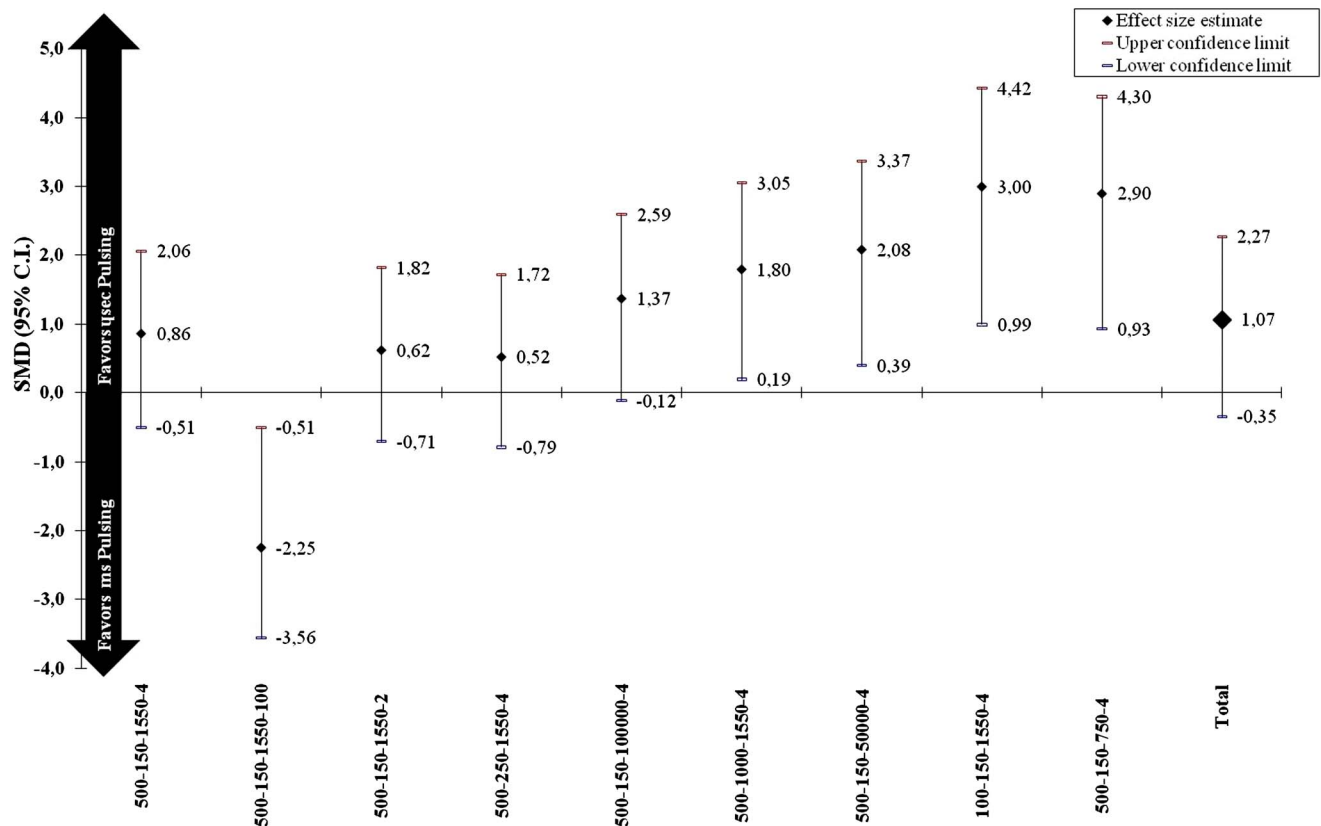

Fig. 6 Standard mean differences between microsecond and millisecond Pulsing. C.I.: confidence interval.
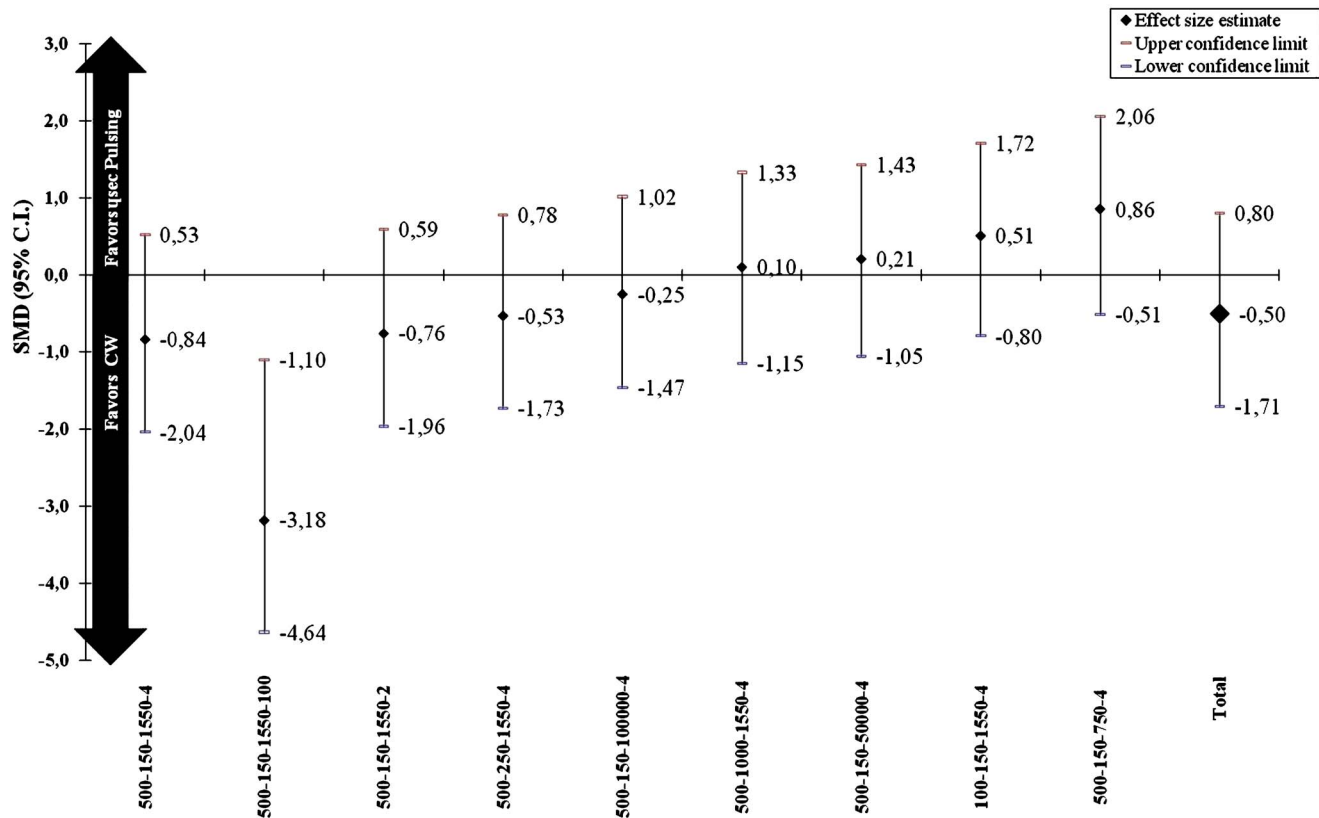
Fig. 7 Standard mean differences between microsecond pulsing and CW. C.I.: confidence interval.

measurements. Each experimental condition was done in quintuplets (five wells in separate plates). The step-by-step procedure is presented in Fig. 2.

2.2 Light Source Specifications

Fibroblast replicates were exposed to three treatments with an LED prototype based on LumiPhase™ technology (OPUSMED Inc. Montreal, Canada) or to a sham light source. The custom-built LED device was designed to irradiate from under the 24-well culture plate, through the transparent floor of every individual well (Fig. 1). This configuration was developed to avoid undue stress and contamination to cell cultures during LED exposure. Also, every well was made of a fully opaque wall to avoid interwell illumination.

A reference condition was determined to be the application of a 630-nm wavelength delivered in a sequential pulsing mode with an irradiance of 8 mW/cm², total fluence of 1,33 J/cm², and treatment duration of 1000 s. The pulsing patterns and time-on and time-off sequences were as follows: pulse duration (PD, time on) 500 μs, pulse interval (PI, time off) 150 μs, 4 pulses per pulse train (PPT), and a pulse train interval (PTI) of 1550 μs. The control/nontreated samples were exposed to sham light with a total fluence of 0 J/cm². Figure 3 presents a schematic representation of the various pulsing parameters employed in this experiment.

Keeping the other reference light parameters constant, two PDs (100 and 500 μs), three PIs (150, 250, and 1000 μs), four PTIs (750, 1500, 50,000, 100,000 μs), and three PPTs (2, 4, and 100) were evaluated. Three comparator conditions were included CW mode, millisecond-domain pulsing, and regular pulsing mode with 50% duty cycle (P50%DC). The duty cycle percentage represents the ratio of illumination on time to off time. Table 1 outlines the details of the experimental conditions.

2.3 Quantitative Determination of Procollagen Type I C-Peptide

Human type I collagen was measured in cell culture supernatants at the selected time periods with the procollagen type I C-peptide enzyme immunoassay (ELISA-PIP) kit, purchased from Takara Bio Inc. (ThermoFisher, Nepean, Canada), according to the manufacturer's procedures. The culture medium used for our experiment did not interfere with the assay for the dilution used for the dosage (1/40).

3 Statistical Analysis

The endpoint for this study was the difference in percent change from T_0 (baseline) at T_3 (72 h) between LED-treated and nontreated fibroblast cells in type 1 procollagen production. To assess the difference between the microsecond-pulsing parameter levels for each parameter, Kruskal-Wallis or Mann-Whitney tests were used. To quantifying the magnitude of the treatment difference, standardized mean differences (SMDs) between the microsecond-pulsing conditions and each of the comparator conditions were calculated. SMDs were calculated using Cohen's d, where d is the difference in group means divided by the pooled standard deviation. A stan-
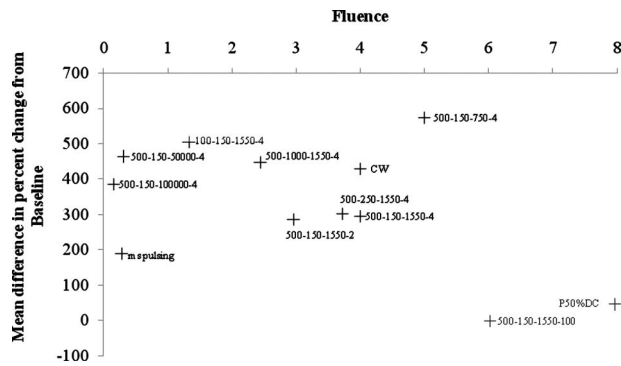
Fig. 8 Relation between fluence and procollagen level (mean difference in percent change from baseline) for each light delivery condition. ms pulsing: millisecond pulsing condition; CW: continuous wave mode condition; P50%DC: regular pulsing mode with 50% duty cycle condition.

Fig. 9 Relation between duty cycle and procollagen level (mean difference in percent change from baseline) for each light delivery condition. The duty cycle percentage represents the ratio of illumination of on time to off time. ms pulsing: millisecond pulsing condition; CW: continuous wave mode condition; P50%DC: regular pulsing mode with 50% duty cycle condition

dardized effect size of 0.2 is considered small; 0.5, medium; and 0.8, large.[14] Correlations were computed with Spearman rho correlation coefficient (two-tailed). The p values were considered significant at $p \leqslant 0.05$. The PASW 18.0 statistical software was used for statistical analyses.

4 Results

Figure 4 depicts type 1 procollagen levels for the microsecond pulsing conditions. As can be noted, a wide range of collagen production responses by fibroblast cells were observed. To assess the relative influence of each microsecond pulsing parameter on collagen stimulation, one level from the preset microsecond pulsing reference condition was modified at a time. With regards to the PD parameter, results showed that when PD was reduced from 500 to 100 μs, the cellular collagen production was largely enhanced. The difference between these two conditions was of 211% ($p=0.12$). The most efficacious PI was the highest tested of 1000 μs. The difference in levels of collagen between this condition and the reference formula was of 152% ($p=0.12$). The analysis also revealed that the best PTI was 750 μs, which produced 279% more procollagen than the reference condition ($p<0.05$). This illumination condition was in fact the best formula to stimulate collagen production by fibroblast cells. Finally, data analysis revealed that reducing PPT from 4 to 2 did not have any significant effect on levels of collagen (9%). The lowest level of collagen production was seen when the PPT parameter was increased from 4 to 100. The difference between the reference condition and the PPT 100 condition was of 296% ($p<0.01$).

The comparator conditions yielded disparate collagen levels: 46% for the P50%DC condition, 190% for the millisecond pulsing condition, and 429% for the CW condition. To quantify the magnitude of the treatment difference between the microsecond pulsing conditions and the comparator conditions, SMD were calculated. It was observed that the microsecond pulsing mode was overall more efficacious than the P50%DC to stimulate collagen production by fibroblasts. With the exception of one condition (PPT100), SMDs were large and all in favor of the microsecond pulsing conditions (Fig. 5). The same pattern of results was seen when microsec-
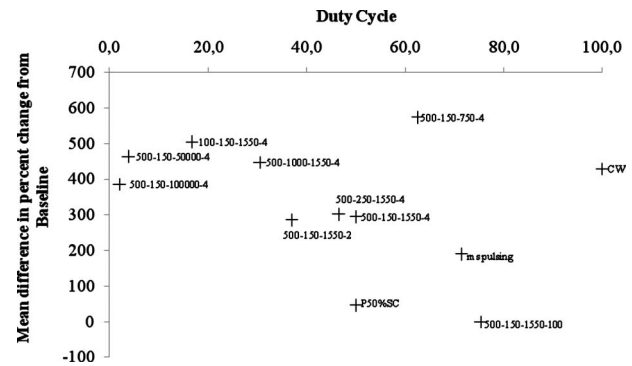
ond and millisecond pulsing modes were contrasted (Fig. 6). For the CW comparator condition, SMD calculations revealed that, overall, the CW delivery mode triggered more collagen secretion by fibroblasts than the microsecond pulsing patterns. However, specific microsecond pulsing patterns (PD100, PI1000, PTI50000, and PTI750) had a more favorable impact on the ability of fibroblasts to produce collagen *de novo* than CW delivery (Fig. 7). Moreover, the SMD between the CW and the best microsecond pulsing condition, namely, the PTI750 condition, was shown to be large.

Correlation analyses revealed that the results were not found to be influenced by exposure time ($\rho=0.064$) (data not shown) or by fluence ($\rho=-0.215$) (Fig. 8). On the other hand, collagen levels were shown to be negatively correlated with the duty cycle (Fig. 9). Overall, the higher the duty cycle, the lower the collagen production ($\rho=-0.313$, $p<0.02$). This correlation was weak and may only partially explain the results. As can be seen in Fig. 9, high duty cycle conditions ($>50\%$) were associated with both the worse collagen production conditions (PD100, P50%DC, and millisecond pulsing), as well as with conditions that yielded high collagen levels (PTI750 and CW).

5 Conclusions

The purpose of this study was to identify the optimal parameters to be used in LED protocols to modulate type 1 procollagen using a model of human primary fibroblast cultured in monolayers. We investigated the impact of various light delivery modes on *de novo* collagen production after three treatments with red light (630 nm, 8 J/cm^2).

Our results suggest that the way light is delivered impacts cellular response. It was observed that low PD (100 μs), PTI (750 μs) and PPT (4), and high PI (1000 μs) were the best pulsing parameter levels to enhance collagen secretion in fibroblasts cells. Future experiments must assess, however, whether combining these factors will lead to the optimal set of parameters to enhance collagen production. Data also showed that specific microsecond pulsing patterns had a more favorable impact on collagen upregulation than the comparator

conditions of millisecond pulsing, P50%DC, and CW illumination modes in the ability of fibroblasts to produce collagen *de novo*. The best microsecond pulsing condition identified was seen when pulse train duration was set at 750 μs, which yielded a large SMD with all comparator conditions.

This work was designed as a first step in the complex undertaking of delineating the impact of emission parameters in LLLT on cellular responses. In this work, although we strived to limit confounding variables in our assessments, some variables varied with conditions. Among these, total fluence, illumination time, and duty cycle were heterogeneous across conditions. Only the duty cycle was found to bear some degree of association with the observed results. In this study, overall, experimental conditions with low duty cycle and low fluence were more efficient in triggering collagen production by fibroblasts. This observation suggests that the amount and time of light emission are important to collagen production by fibroblasts. Our results are in line with the results from other studies suggesting that cell viability and mitochondrial activity is more efficient with low LLLT total doses.[15] Certainly a minimal exposure time per treatment is necessary—of the order of several minutes rather than only a few seconds—to allow activation of the cell machinery. However, the data do not appear to corroborate the law of reciprocity, which states that a certain biological effect is directly proportional to the total energy dose irrespective of the administered regime. This law may not apply to photobiomodulation processes.

In this study, we reported data from the 72-h postbaseline time point following three LED treatments. However, there are some indications in the literature that cellular responses following light irradiation are time dependent. For instance, a recent study suggested that responses such as ATP viability can be observed directly (1 h) after the irradiation, whereas other responses such as cell proliferation require at least 24 h before the true effect can be observed.[16] Our own laboratory experience shows that physiological cyclical patterns of procollagen type I upregulation are emphasized[17] by LED treatments every 48 h. Results may thus differ at other time points. Other limits of the presented experiment include the fact that we tested only the 8-mW/cm^2 irradiance and the 630-nm wavelength. Results may prove to vary with other wavelengths and irradiance, as well as with other light variables, such as the number of, and interval between treatments.

The optimal parameters identified in this study seem to imply that cells require time/pauses to absorb and handle photons. Targeted molecules/cells may have—similarly to thermal relaxation times (TRTs) in selective photothermolysis—their own TRT and cycle of collagen production, periodically turning the factory on and off. In addition, over- or understimulation of these endogenous processes may hinder specific cell signaling pathways. Too long a pulse may produce cellular exhaustion, whereas too short a pulse may deliver insufficient energy for a biologic effect to occur. Indeed, the way the energy is delivered over a period of time is an important determinant of the efficacy of LED therapy within a limited window of stimulation, as described in the Arndt-Schultz curve.[18]

Current evidence suggests that the cascade of events leading to photobiomodulation effects by red to near-IR illumination is initiated by the antenna molecule mitochondrial cytochrome c oxidase.[1] Respiration in the mitochondria can be inhibited by nitric oxide (NO) binding to cytochrome *c* oxidase, which competitively displaces oxygen and affects cell metabolism. Excess NO binding is associated with inflammatory processes, cell damage, and apoptosis. Light absorption dissociates NO, enabling cellular respiration to resume and normalization of cell activity, ultimately triggering biomolecular processes. Pulsed light delivery might favorably enhance this cellular strategy. Short and intermittent light emission might enhance NO dissociation, therefore augmenting mitochondrial energy production and cellular activity leading to collagen production.

Our experimental findings demonstrated that identification of the proper treatment parameters for a specific cell line and biological process is crucial to achieve optimal photobiostimulation. More importantly, our results substantiate that collagen upregulation can be achieved at low irradiance and low fluence. Hence, one must not underestimate the importance of dose rate and pulse structure when using LLLT sources such as LEDs to stimulate cellular processes. Results from this study may shed some light on conflicting study results, demonstrating both positive and negative effects, and why the efficacy of LLLT remains controversial. Finally, the controversy surrounding LLLT may be fed by misuse of the terminology. By definition LLLT uses a low level of light and does not apply to all light therapies.

Future studies to further define these effects and to investigate whether these results can be replicated in animal models are warranted. Further studies should also ascertain whether our study findings can be transposed to relevant clinical applications. Our laboratory intends to continue assessing the importance of LED parameters to identify the best possible parameters to be used in LED protocols.

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