IN VIVO HUMAN DERMAL COLLAGEN PRODUCTION FOLLOWING LED-BASED THERAPY: THE IMPORTANCE OF TREATMENT PARAMETERS

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Abstract

Background and Objectives: Actual non-ablative thermal technologies induce micro-injuries and tissue repair mechanisms to enhance collagen production. Conversely, the improvement in skin appearance following LED-based therapy results from increased collagen production by dermal fibroblasts without thermal injury. The assumed effect is the selective absorption of light by intracellular antenna molecules which trigger specific gene expression leading to phenotypic dermal collagen deposition (skin rejuvenation).

Study Design / Materials and Methods: Quantitative measures of the collagen synthesis rate in the skin can be determined by the aminoterminal propeptide of type III procollagen (PIIINP) levels in suction blister fluid using radioimmunoassay. Three weekly treatments were performed on 10 healthy volunteers (5 adjacent 3 X 4 cm spot tests) on the volar aspect of both forearms using the LumiPhase-R™ LED system at 660nm. Two suction blisters were raised in every test area (20 blisters), 72 hours after the last (12th) LED treatment. Blister fluids were collected and analyzed.

Results: Twelve treatment sessions at high fluence (5 J/cm2) and high irradiance (60 mW/cm2) induced an increase of 50% ($p < 0.01$) in the type III pro-collagen production rate compared to non-treated control site with sequentially pulsed LED therapy, whereas the increase was only 29% ($p < 0.003$) with CW mode. Similarly, for the same number of treatments, the same pattern occurred at low fluence (0.1 J/cm2) and low irradiance (5 mW/cm2) inducing an increase of 76% ($p > 0.05$) in the type III pro-collagen production rate
compared to non-treated control site with the sequential pulsing mode, whereas the increase was only 38% ($p = 0.05$) with CW mode. No significant differences were found between the high fluence (right arm) and low fluence (left arm) side, nor between the high and low irradiance parameters being tested.

Discussion: The pulsing of LED light to the treated area seems to be an important part of the equation to trigger cytomodulatory effects within dermal fibroblasts in order to induce

collagen production and deposition in the ECM (extracellular matrix). The sequence in which the proprietary sequential pulsing occurs is another key component to producing optimal photobiochemical reactions that likely take place in the mitochondrial wall and leading to signal transduction and amplification, ultimately enhancing specific gene expression.

Conclusion: This study demonstrates that non-thermal skin irradiation by a deeply penetrating wavelength (660 nm) LED device induced a significant increase in the dermal procollagen production rate, especially when proprietary sequential pulsing was used. The notion of sequential pulsing mode, we believe, provides cells cyclical resting intervals between light exposures which may enhance an already successful biological response achieved with a continuous wave mode (CW).

Introduction

Healthy collagen gives the skin its softness, resiliency, suppleness as well as its ability to repair itself. Low Level Laser Therapy (LLLT) has been used in phototherapy since the1970s to promote collagen production and speed up wound recovery. Experiments using light emitting diodes (LEDs) to invigorate in vitro fibroblast proliferation, collagen production and angiogenesis suggest faster wound healing and promotion of collagen metabolism (Saperia *et al.* 1986; Wheeland 1993; Yu *et al.* 1994). However, proof of concept of LLLT for collagen enhanced production and tissue remodelling remains to be established (Saperia *et al.*
1986; Nussbaum *et al.* 1994; Whelan *et al.* 2001). An alternative LED light source could also induce such photobiochemical reactions, upregulating collagen metabolism if key parameters are used. Literature demonstrates the critical parameters to consider in the design of a comprehensive approach to enhance dermal collagen production using a non-ablative, non-thermal light source. For instance, specific wavelengths were found to increase growth characteristics in fibroblasts (Webb *et al.* 1998), and the fluence as well as the irradiance reaching the cell are important to achieve a significant positive clinical outcome from light therapy (Bihari and Mester, 1989; Yu *et al.* 1994; Skinner *et al.* 1996; Webb *et al.* 1998;
Almeida-Lopes *et al.* 2001). However, to the best of our knowledge, no in vivo experiment has been reported so far on the superior ability to trigger dermal collagen using pulsed versus CW light. Herein, we report *in vivo* dermal collagen production in suction blister fluid following light-based therapy using a non-ablative, non-thermal 660nm LED light source. Combination of either high or low fluences, high or low power densities and proprietary pulsing versus continuous wave modes were tested.

Materials and methods

Ten subjects (healthy volunteers), ranging from 31 to 52 years of age, undergoing 12 treatment sessions (three weekly treatments) over a four-week period were selected for LED irradiation. Five adjacent test areas, each measuring 3 x 4 cm, were determined on the volar aspect of both forearms and studied parameters were distributed in adjacent but individual test areas. Two suction blisters were raised in each of the test areas (20 blisters), 72 hours after the last treatment, and blister fluids were analyzed. Harvesting of

blister fluid was performed 72 hours after the 12th treatment for proper dosage of collagen assessing aminoterminal propeptide of type III procollagen (PIIINP) level by radioimmunoassay.

The LED parameters were as follows:
Left Forearm: Fluence: 0.1 J/cm2
Right Forearm: Fluence 5 J/cm2
Device description:

No pre-or post-treatment preparations were used. At 72 hours after the 12th treatment session, skin suction blisters were raised in the control and the treated areas as previously described (Kiistala *et al.* . Skin suction blister were raised in the control and tested areas. Five adjacent dual suction cups containing two holes (5mm) each were set simultaneously to both right and left forearms (250-300mmHg negative air pressure through each cup). Blisters were raised from the volar aspect of the forearm, two blisters per test area, for a total of 20 blisters per participant (10 on each arm). During a period of 40-270 minutes, epidermis and dermis split at the dermo-epidermal junction and dermal interstitial fluid accumulated in the space that slowly became a blister.
The blisters were then gently punctured with a G18 needle and the blister fluid (10-120mL) collected with a sterile pipet (P20 PipetmanTM), then transferred to a 1.5 mL tube (EppendorfTM). Samples were quickly frozen on dry ice for radioimmunoassay.
Levels of PIIINP (Type III collagen) measured 72 hours after the last treatment:
The blister fluid was analysed for the concentration of the aminoterminal propeptide of the type III procollagen (PIIINP), this level being indicative of the skin type III collagen production rate (Jensen *et al.,* 1990) which is fairly representative of dermal procollagen type I levels. The levels of the PIIINP concentration in the treated and untreated areas were compared to determine the effect of the different types of irradiation. The concentration of the suction blister fluid was analysed using a radioimmunoassay (Orion Diagnostica, Finland). **Suction blister fluid harvesting.**

Statistics
The statistical analyses were performed using t-test due to the relatively small number of observations. Statistical significance was accepted at the standard 5% level.

Results
Biochemical analysis 3 days after the twelfth treatment session at high fluence (5 J/cm2) and high irradiance (60 mW/cm2) demonstrated an increase of 50% ($p < 0.01$) in the type III pro-collagen production rate compared to non-treated control site with sequentially pulsed LED therapy, whereas the increase was only 29% ($p < 0.003$) with CW mode. Similarly, with an identical number of treatments, a similar pattern of procollagen production also occurred at low fluence (0.1 J/cm2) and low irradiance (5 mW/cm2) with an increase of 76% ($p > 0.05$) in type III pro-collagen production rate when compared to non-treated control site with the proprietary sequential pulsing mode, whereas the increase was only 38% ($p = 0.05$) with the CW mode.
The data obtained for both high fluence (right arm) and low fluence (left arm) as well as

between the high and low irradiance parameters were significantly higher than the non-treated control sites but not substantially different from one another.
LOW FLUENCE – LOW IRRADIANCE
HIGH FLUENCE – HIGH IRRADIANCE

Discussion
This study was performed on an unexposed area (inner forearm) in accordance to the suction blister protocol. Therefore, skin was not photoaged at the study site. Cellular threshold irradiance, once reached, would induce an increased physiological stimulation for collagen synthesis especially if the cellular integrity has been alter prior UV insults (cumulative photodamage in exposed areas like the face). The magnitude of light biostimulation would depend on the physiological condition of the cell at the moment of irradiation. LED therapy enhances collagen metabolism. Superior type I procollagen production was obtained at 660nm, when compared to 635 and 805 nm, using human primary fibroblast monolayers (Barolet *et al.* preparation). At 660 nm, light wave was found to reach through the whole papillary layer for maximum collagen production (Webb *et al.* 1998). Herein, combinations of key parameters using 660 nm light emitting diode (LED) collagen for photoinduction were tested *in vivo* Unlike non-ablative thermal technologies where wound healing must take place, LED light does not injure the skin. There is no need for an acute inflammation phase followed by matrix and cell proliferation phase which eventually ends by a remodelling phase. Rather, non-thermal light activation of cellular components at a proper wavelength was found to induce metabolic modulations correlating with tissue response (Smith, 1981; Anderson *et al.* 1981; Wheeland *et al.* 2001). Recently, gene expression profiles of human fibroblasts irradiated by low-intensity red light reported direct and indirect activation of pathways related to cellular proliferation and metabolism (Zhang *et al.* 2003). However, despite several promising studies suggesting the potential of LED for skin rejuvenation, proof of concept for this light therapeutic potential remains to be established. Aside from wavelength, several important parameters must be combined for optimal LED collagen upregulation.
The fluence (J/cm2) or total dose of energy released over a set amount of time is an important determinant of efficacious LED therapy within a limited window of stimulation as described in the Arndt-Schultz curve (Sommer *et al.* 2001). Threshold irradiance or power density (mW/cm2), or intensity per unit area must also be reached to promote collagen production. One must consider that biologically, fluence and irradiance are independent variables. For instance, one must bear in mind that for equal fluence delivered, irradiance values under the threshold point, even under prolonged irradiation time, could never result in biostimulatory effects. In this study, increased collagen secretion was found with both high and low fluences and also using high and low irradiances. This may be explained by the fact that only one dosage was performed at the end of the study. Indeed, cyclical collagen fluctuations throughout the 12 treatment sessions and the cumulative effect of this therapy were not monitored during this protocol. In fact, the final PIIINP reading represents fibroblast ability to secrete collagen at a given moment. It is somewhat like an actual snapshot (picture) of the collagen metabolism in the dermis. It does not give information on the cumulative status or

collagen accumulation end point in the ECM (extracellular matrix). It is hypothesized from our *in vitro* that the higher the irradiance utilized for a short period of time (short treatment session i.e. 160sec), the higher the fibroblast stimulation in the first few treatment sessions. In fact, it has been shown *in vitro* in our lab) that decremental peaks of the procollagen secretion rate were progressively measured during the whole treatment cycle when using high irradiance (60 mW/cm2) and high fluence (5 J/cm2) (Barolet *et al.* preparation) (see graph).

Percent Change in Procollagen type I Secretion vs Control Over 11 Treatments for a 64 year- old female (in vitro study using human reconstructed skin)

The stimulation decreases progressively over the 12 treatment sessions meaning that the final measurement would show less collagen stimulation at the time. Hence, this final reading does not reflect the total amount of collagen accumulated in the dermis that is expected to bring morphological improvements in skin texture. With quicker fibroblast initial response, faster clinical results (with more *de novo* collagen deposition) are then more likely to occur. Conversely, low irradiance (and low fluence) would show higher collagen stimulation after the 12th treatment session since the fibroblast seems to only engage in the stimulatory process. The present study shows a trend towards inducing more type lll procollagen with low irradiance (with low or high fluences) (55.5%) compared to high irradiance (with low or high fluences) (49.5%)—however, no statistical difference was obsered between those two study groups. Moreover enhanced fibroblast activity lasts typically 30 days, reaching a maximum approximately 10 days after treatment (Ross *et al.* . Therefore, additional blister fluid measurements along the treatment sessions and mid-term after the last treatment would have been very useful to demonstrate the importance of high/low intensity and/or high/low fluence chronologic collagen build- up potentials. Finally, as this study demonstrates, pulsing LED light (vs CW mode) proved to be a significant parameter likely due to the periodic relaxation time it allows the fibroblast to avoid cellular exhaustion. **A proprietary sequential pulsing mode would provide molecular and cellular resting intervals. It is hypothesized that pulsed LED light, when compared to continuous waves (CW), would provide rest to the fibroblast thereby preventing cell exhaustion (Barolet *et al.*, in preparation). A) The threshold irradiance (mW/cm2) or intensity per unit area must be reached to activate the fibroblast. B) During this experiment, the exposure duration (time on) was a closely monitored factor. C) The innovative component is the importance of the pulse interval that would provide optimal molecular relaxation (time off). D) In addition, a longer downtime between predetermined number of pulse intervals (<10) leading to successive inter-pulse train intervals would provide a resting phase for treated fibroblasts – or cellular relaxation time - assuming that inter-pulse train interval > pulse interval. This, alternatively, may give the fibroblast more time to secrete type I collagen in the ECM.** This *in vivo* further adds to the growing body of evidence reporting the efficacy of LED therapy and emphasizes the importance of using the right combination of parameters to maximize collagen secretion in the dermis. In this experiment, sequential pulsing proved to be of significant value compared to CW mode in inducing *de novo* synthesis. While clinical results are not always easy to quantify, they

nonetheless appear real. It is also assumed that additional treatments promote collagen deposition in a cumulative manner until a steady state is reached in the dermis. Continued low intensity LED irradiation could then be used as part of a maintenance treatment strategy. Newer LEDs may provide more specific improvements of greater absolute magnitude if light parameters are optimized. As the mechanisms underlying nonablative non-thermal therapy are better understood, these treatments may be adapted to exploit their full photobiochemical potential and ultimately lead to the expected clinical outcome.

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