Ablative and nonablative skin rejuvenation procedures with lasers and intense pulsed light (IPL) systems have become very popular for photorejuvenating sun- and chronologically aged skin. Both procedures demand deposition of heat damage in the upper dermis. A new generation of light-emitting diodes (LEDs) with laser-like wavebands and phototherapeutically useful output powers are now reported to be successful for noninvasive athermal photorejuvenation of age-damaged skin. This study was designed to examine the ultrastructural changes induced by irradiation of human skin with visible red LED energy. Six adult male volunteers who satisfied all study criteria had the skin over their fibula irradiated once per week for 8 weeks with a visible red LED-based system at an irradiance of 105 mW/cm², 15 min/session and a radiant flux of 94 J/cm². Skin punch biopsies taken from each subject after the second and eighth treatment sessions were routinely prepared for transmission electron microscopy (TEM) and were examined under an electron microscope. After the 2nd session, the skin showed normal undamaged tissue with slight interstitial edema and vimentin filaments notable in fibroblasts. After the 8th session, the number of fibroblasts in the dermis had increased with numerous vimentin filaments in their cytoplasm, and a mild inflammatory infiltration could be seen. The ultrastructure of human skin after 8 LED phototherapy sessions showed no damage-related abnormalities. Mild athermally-mediated inflammation was seen together with an increased fibroblast count and enhanced metabolism, which could be related to the enhanced synthesis of collagen and which in facial skin would probably result in an improved skin appearance.

Key words: Fibroblast, LED phototherapy, neocollagenesis, photobiomodulation, vimentin granules, vimentin fibrils

Introduction

Skin rejuvenation using ablative or nonablative techniques with lasers and intense pulsed light (IPL) systems, has become extremely popular over the past few years.(1) However, the ablative approach is associated with severe side effects and a prolonged patient downtime while nonablative resurfacing with single wavelength light sources has proved less effective than hoped(2,3) although combinations of wavelengths have recently proved much more effective.(4,5) Both ablative and nonablative skin rejuvenation methods demand the deposition of thermal damage in the upper dermis. In cases with less severe photo- and chronological ageing sequelae, it is possible that a much less invasive and virtually athermal reaction could be very effective, based on the photobiomodulation of target skin cells which leads to the enhanced deposition of collagen and related materials.

Athermal low reactive-level laser therapy (LLLT) has been successfully used for a variety of complaints and conditions,(6,7) based on its effects on the neural network, lymphatic and blood circulatory systems, and it has been shown to have wavelength-specific effects on fibroblasts,(8) mast cells(9) and the blood vasculature system.(10) LLLT systems could therefore be used to deliver the required noninvasive energy to photoaged skin, but they are still comparatively expensive, and are capable of treating only a single point per irradiation. This makes treating large areas, such as in photorejuvenation of the entire face, very time- and labour-intensive. A new generation of narrow waveband light-
emitting diode (LED) based devices now offers an alternate less expensive phototherapeutic source, capable of treating large areas during a single automated irradiation therapy session. In July of 2003, a visible red LED phototherapy system (Omnilux® revive®), Photo Therapeutics, Ltd., Fazely, UK) was approved by the Unites States Food and Drug Administration (FDA) for skin rejuvenation.

Two reports have recently appeared on the effective macroscopic treatment by LED phototherapy,\(^{(11,12)}\) but nothing has been published to date evaluating the efficacy of this method at the microscopic level. This transmission electron microscopy (TEM) study was designed to examine the morphology of red LED-irradiated human skin \textit{in vivo}, with particular emphasis on photoinduced changes in fibroblasts.

\textbf{Subjects and methods}

\textbf{Subjects}

Six healthy adult volunteers (mean age 31.7 years, range 35-48 years) were recruited for this study. All subjects were Japanese with Fitzpatrick skin type III. Exclusion criteria for this study included chronic illness, cutaneous contact dermatitis, atopic dermatitis, stasis dermatitis, history of scar formation, vascular disease, stasis dermatitis, history of poor wound healing, or any form of cutaneous disease. This study was approved by the Ethics Committee of the Japan Equestrian Federation for Sports Medicine Research. Before the study began, the study’s objectives and requirements were explained to, and written informed consent was obtained from, each volunteer.

\textbf{Phototherapy protocol}

Phototherapy was delivered with a red LED-based free-standing unit (Omnilux® revive®), Photo Therapeutics Ltd., Fazely, UK). The treatment head comprises an array of visible red LEDs \(\lambda = 633 \pm 3 \text{ nm}, \) irradiance of 105 mW/cm\(^2\), 15 min/session, radiant flux of approximately 94 J/cm\(^2\), active area approximately 22 cm x 18 cm) precisely mounted in 4 articulated panels which enable the treatment head to follow the contour of the area to be treated, in this case the lateral calf. The head is attached to an articulated arm and is set up with the LED panels approximately 1.5 cm from the target tissue. The system is illustrated in Figure 1. The desired parameters were selected on the base unit control console, and, after the subject had donned appropriate protective eyewear, the system was activated.

In each subject, an area was chosen on the medial calf over the fibula. Each area was treated with the LED-based system using the above parameters, once per week for 8 weeks, 15 min per treatment session.

\textbf{Morphological Assessment}

Three millimetre skin punch biopsies were obtained from each subject after the second and eighth treatment sessions. The specimens were fixed in 2.5% glutaraldehyde and were post-fixed with 1% osmium tetroxide. The tissue samples were dehydrated through a graded ethanol series and were embedded in Epon 812. Ultrathin sections were cut using an Ultracut N ultramicrotome (Reihert-Nissei) with a diamond knife, were stained with oolong tea extract (OTE) for connective tissue staining following the method of Sato \textit{et al.},\(^{(13)}\), and were further stained with uranyl acetate and lead citrate. The sections were examined in an electron microscope (75 kV, Hitachi H-7500; Hitachi, Tokyo, Japan).

\textbf{Results}

All six subjects completed the trial. All subjects noted a very slight sensation of warmth in the treated area during phototherapy, but it was not at all uncomfortable, and was totally pain-free. No subject reported any adverse effects, and no erythema, immediate or delayed, was noted during or after any session.

The photomicrography was very similar for all subjects. One representative sample is shown from the same subject for each assessment point. Figure 2 is a typical tissue specimen after the 2nd treatment session. The morphology of the skin was basically normal, with a few fibroblasts seen. Slight perivascular and interstitial edema was noted, with some mast cells in the act of mild degranulation. After the 8th treatment session (Figure 3), more lymphocytes, mast cells and fibroblasts were noted around the capillaries compared with after 2 treatments, with some partially degranulated mast
Fig 2: Transmission electron microscopy of irradiated dermis after the 2nd irradiation. Basically normal morphology can be seen, with no damage-related changes observed either in capillaries (Ca) or in fibroblasts (Fb). A few mast cells (Ma) are also seen. Magnification X 3,000.

Fig 3: After the 8th treatment session, many fibroblasts (Fb) and lymphocytes (Ly) are seen surrounding the capillaries (Ca) in the dermis, compared with findings after the 2nd irradiation. An enlarged perivascular interstitium can also be seen and mast cells (Ma) can also be observed. No damage-related morphological changes are noted either in the capillaries or in the fibroblasts. Magnification x 2,000.
cells. An enlarged perivascular interstitium was also observed. As seen after 2 treatments, the morphology was basically normal with absolutely no signs of any damage-related morphological changes but with some signs of a mild inflammatory infiltration.

TEM photomicrography of unirradiated skin shows a typical fibroblast with collagen fibers both in longitudinal- and cross-section seen in the extracellular area (Figure 4). Golgi complexes, rough endoplasmic reticulum and mitochondria were seen in the cytoplasm, but few vimentin granules or filaments were noted. After the 2nd treatment session (Figure 5), more mitochondria were present in the cytosol of fibroblasts with a significant increase in vimentin filaments compared with the unirradiated skin. After the 8th session (Figure 6), there was a dramatic increase in the number of vimentin filaments (arrows) compared with the unirradiated skin.

**Fig 4:** A normal fibroblast in the dermis of an unirradiated specimen from one of the subjects is shown, with few mitochondria and rough endoplasmic reticulum visible. A few vimentin filaments (arrows) can be seen. Magnification x 12,000.

**Fig 5:** A fibroblast in a specimen after the second therapy session at high magnification (x 30,000) shows increased numbers of vimentin filaments (arrows), and a higher number of mitochondria compared with unirradiated fibroblasts. Note that the intranuclear chromatin has located at the nuclear membrane compared with the situation in Fig 4, suggesting increased signalling between the nucleus and the cytoplasm.
of vimentin filaments, with increased numbers of somewhat more electron dense mitochondria. Figure 7 shows a higher magnification of an endothelial cell after the 2nd treatment session, with increased numbers of vimentin filaments and granules, similar to the findings in fibroblasts. However, unlike fibroblasts, the number did not increase further in specimens after the 8th session, but remained more or less constant (data not shown).

Discussion

LED phototherapy used for skin rejuvenation is reported to show mild to medium improvement even in wrinkles which should probably be the prime target of combined wavelength nonablative skin rejuvenation. In all patients, although wrinkles were not removed completely, the overall appearance of the skin was younger, firmer and plumper. Until the present study, no study had ex-

Fig 6: A fibroblast from a specimen after the 8th therapy session. More mitochondria are seen in the cytoplasm which are now electro-dense, and some can be seen in the process of division to increase mitochondria numbers even more. Vimentin filaments (arrows) are significantly increased in number. (Magnification x 30,000)

Fig 7: Compared with unirradiated cells, endothelial cells from specimens after the second therapy session show an increased number of vimentin filaments in the cytoplasm (arrows), similar to findings seen after the 8th irradiation session. (Ca, capillary; Magnification x 30,000)
amined the morphological histology behind the macroscopic amelioration and rejuvenation of target skin with LED phototherapy at 633 nm.

The choice of skin on the calf as the target was dictated by the necessity to take punch biopsies of the irradiated tissue, and in males the legs are nearly always hidden so full compliance with the study was not a problem for them. A minor limitation of this study is the fact that the target tissue is on the calf, and not on the face of the subjects.

Although it could be argued that the morphology of skin on the face and on the leg has some differences, particularly in epidermal thickness and in the density and type of hair, the dermis in both areas has a similar architecture, and most importantly, the fibroblasts are the same in both sites. In any event, the main aim of this study was not to examine macroscopic changes in the appearance of the skin, but to elucidate the morphological cellular response to visible red LED energy.

Fibroblasts are important cells in the dermis, not only because they secrete collagen and elastin fibers, but also because they are one of the primary regulatory cells in the dermis. In addition to synthesizing dermal fibrous components, fibroblasts maintain these fibers and the homeostasis of the dermal architecture through ground substance synthesis.

Visible red light has a long history in selectively activating cells, with the redox chain of mitochondria as the principal chromophore. Fubini et al. first presented these findings in the late 1800’s, before the turn of the last century, so such knowledge is not exactly new. During the latter part of the 20th Century, large volumes of work from Karu and colleagues in Russia, Dyson and colleagues in the UK and Lubart and colleagues in Israel have greatly clarified the role of visible red waveband in cells in general, and in fibroblasts in particular. Rigau et al. showed that, compared with unirradiated controls, fibroblasts irradiated with red light achieved confluence significantly faster. Moreover, the fibroblast monolayer was much better organized in the experimental groups, with the fibroblasts oriented well with each other, the ideal situation in achieving a good-looking skin in photorejuvenation.

However, most of those previous studies were in vivo and not much work has been done on fibroblasts in vitro with the very low irradiances or power densities produced by LEDs. To the best of our knowledge, this is the first study to use TEM to examine the morphological ultrastructure of the dermis following serial treatment with 633 nm LED phototherapy.

At the level of fibroblasts, our results have demonstrated an enhanced level of metabolism which increased in a treatment-dependent manner, as seen by the more abundant mitochondria and vimentin in the cytoplasm of irradiated compared with unirradiated fibroblasts. The increase in the number of mitochondria, the power houses of the cell, are indicative of the greater energy demands which go hand in hand with increased metabolism. In the study by Rigau and colleagues, an increase in glucose consumption and a highly significant increase in cellular ATP were seen in HeNe-irradiated fibroblasts compared with unirradiated controls (p < 0.001), which is in accordance with the data in our study.

Vimentin, a developmentally regulated member of the intermediate filament protein family, is believed to play a part in communication and transport between the cell surface and nucleus by interconnecting those two organelles. In addition, vimentin copolymerizes with appropriate desmins to form the constituents of connective tissue, i.e., collagen. The presence of increased quantities of vimentin in this in vitro trial could therefore be translated into increased collagen synthesis following low incident doses of 633 nm red light, as demonstrated in vitro in the trial by Rigau and colleagues. The appearance of lymphocytes after the 8th irradiation suggests a mild but extended athermally mediated inflammatory response, most probably induced by photocaccelerated mast cell degranulation, which has been associated with 633 nm light both in vitro and in vivo. Inflammation is recognized as a necessary stage of the wound healing process, leading to proliferation and remodeling, both of which have been associated with successful skin rejuvenation.

Apart from the irradiation site-related limitation mentioned above (skin on the leg versus on the face), this study has a further limitation in that there was no long-term follow-up period after the final irradiation, and staining with haematoxylin and eosin and elastin van Giesen reagent was not performed which would better demonstrate collagen formation during the remodeling period. However, the positive preliminary findings at the TEM level have been extremely encouraging and these limitations will be addressed in future studies on human facial skin with long-term macrophotography and histological follow-up.

Conclusions

In conclusion, we successfully demonstrated at the cellular and subcellular levels that repeated irradiation of human skin with a 633 nm LED-based phototherapy system has no adverse effects of a photothermal or any other nature on tissue morphology. We further showed that successive treatment with 633 nm light increases the number of fibroblasts in the treated area, raises the metabolic level of endothelial cells and of fibroblasts (the latter in a treatment-dependent manner), dramatically increases the numbers of vimentin and filaments in
fibroblasts, and induces the infiltration of lymphocytes into the treated area, which suggests a mild athermally mediated inflammatory reaction. Taking these results into consideration with previously reported in vitro studies on accelerated collagen production in 633 nm irradiated fibroblasts, the combination of these factors probably leads to enhanced collagen synthesis and remodeling, which is one of the major goals of athermal LED phototherapy for skin rejuvenation.

References

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