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Low-Level Red Plus Near Infrared Lights Combination Induces Expressions of Collagen and Elastin in Human Skin *In Vitro*

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Abstract

Objective: Light therapy has attracted medical interests as a safe, alternative treatment for photo-aging and photo-damaged skin. Recent research suggested the therapeutic activity of red and infrared (IR) lights may be effective at much lower energy levels than those used clinically. This study was to evaluate the efficacy of low-level red plus near IR light emitting diode (LED) combination on collagen and elastin and ATP production.

Methods: Human dermal fibroblasts or skin tissues were irradiated daily by red (640 nm) plus near IR (830 nm) LED lights combination at 0.5 mW/cm^2 for 10 minutes (0.3 J/cm^2). qPCR, ELISAs or histology were used to determine the gene and protein expressions. Fluorescent measurement was used to assess cross-links of collagen and elastic fibers. ATP production was evaluated by ATP assay.

Results: Treatment of human fibroblast cell cultures with low level red plus near IR lights combination was found to significantly increase LOXL1, ELN and COL1A1 and COL3A1 gene expressions as well as the synthesis of the procollagen type I and elastin proteins. Treating human skin explants with low level red plus near IR lights combination similarly induced significant increases of the same genes expressions, type III collagen and elastic-fiber formation and cross-links. ATP production was increased in human dermal fibroblasts after red plus near IR lights combination treatment.

Conclusion: Low level red plus near IR lights combination stimulated the production of collagen and elastin production associated with anti-aging benefits. These findings suggest that low level red plus near IR LED light combination may provide an effective treatment opportunity for people with photo-aged skin.

Keywords: Skin physiology/structure; Genomic/Proteomic/ELISA/Cell culture; Photobiomodulation

Introduction

Collagen and elastin are two of the primary structural components of the human skin dermis and are the subjects of most anti-aging research and efforts for aesthetic-anti-aging strategies pertaining to the skin [1,2]. In the normal aged skin, there are decreased level of collagen synthesis and fragmented elastic fibers. Disorganized collagen fibrils and the accumulation of abnormal elastin-containing materials were found in the photo-damaged skin [1].

Low-level light therapy (LLLT) or photobiomodulation (PBM) is the application of non-thermal light to modulate tissue functions, such as skin rejuvenation and tissue regeneration. Since the light's wavelength determines the depth of tissue penetration [3,4], red to IR wavelengths (600-1000 nm) have been used for skin rejuvenation [5] that are absorbed by the mitochondria and may trigger intracellular photo-biochemical reactions to increase adenosine triphosphate (ATP) production, elevate reactive oxygen species (ROS) generation, induce transcription factors and collagen metabolism [6-9].

Red light is the deepest skin penetrating visible light to target dermal structures (2–3 0.55 mm), such as adnexa and fibroblasts [3,10]. When red light (628 nm at 0.88 J/cm²) irradiated human fibroblasts, it stimulated cell growth by directly upregulating the genes related to cell proliferation and inhibition of cell apoptosis [11]. Red light could increase type I procollagen in fibroblast cultures [12] and decrease matrix metalloproteinase-1 (MMP-1) levels in human reconstructed skin tissues at 44 J/cm² (660 nm) [9].

Near IR light, which is a portion of IR-A wavelength range, may penetrates the skin between 5 and 10 mm at the depth of around 1.2 mm [340]. Low doses of IR radiation have demonstrated therapeutic effects on wounds, ulcers and cellulite [13-15]. Near IR (830 nm) at 1.3 J/cm² improved wound healing better than 3 J/cm² [16]. IRA radiation (950 nm, 0.53 J/cm²) could stimulate fibroblast proliferation *in vitro* [17]. Near IR at low doses (810 nm, 1–10 J/cm²) did not induce reactive singlet oxygen and hydroxyl radicals' generations but stimulated therapeutic effects by activating NF- κ B pathway [18] whereas IR at high doses (>120 J/cm²) caused highly reactive singlet oxygen and hydroxyl radicals' generations and pathological effects by co-activating the AP-1 pathway [19].

Several clinical studies have demonstrated that combination of red and IR LED light therapy is efficacious to treat photoaging skin. Red and near IR LED light therapy (633 nm at 126 J/cm² and 830 nm at 66 J/cm², respectively) had 25%–50% improvement in photoaging scores in 52% of subjects by week 12 [20]. Red (633 nm, 70 mW/cm², 30 min for 126 J/cm²) and near IR (830-nm, 55 mW/cm², 20 min for 66 J/cm²) LED light therapy resulted in 50% improvement of the signs of photoaging in 68% of subjects [21]. Subjects treated by red (611-650 nm, ~9 15.1–16.8 J/cm²) or broadband polychromatic (570-850 nm, 15.5 49.3–51.4 J/cm²) light demonstrated significant improvement in skin feeling and complexion, roughness, and collagen density by collagen ultrasonography scans [22]. Moderate to great clinical improvement of skin after six months of broadband (420-1100 nm, 20 J/cm²) pulsed

light was in 77% of subjects with significant increases of collagen (51.33%) and elastic (44.13%) fibers in facial skin after 6 months [23].

Recent research suggested the therapeutic activity of red and IR light may be effective at much lower energy levels than those used clinically [22,24]. Weiss et al [25] showed clinical improvements in photodamaged skin when used 0.1 J/cm² to treat every other day with 590 nm pulsed LED light for 4 weeks. They also showed increased collagen in the papillary dermis of 100% of post-treatment specimens (N=10) and a 28% (range: 10%–70%) average increase in staining density with anti-collagen I antibodies. Since only few *in vitro* studies have investigated the effects on extracellular matrix components by low-level red (640 nm) and near IR (830 nm) LED lights, the purpose of the current study was to examine the effects of low-level (0.3 J/cm²) red plus near IR LED lights combination for collagen and elastin induction in human adult skin fibroblasts *in vitro* and human skin explants *ex vivo*.

Methods

Red and Near IR LED light board setup

LED light boards were designed for the usage on top of cell culture plates with average height of lights at 18 mm above the bottom of each well. The peak wavelengths of red and near IR light were 637 nm and 830 nm, respectively. The light power used was 0.5 mW/cm² measured by a light sensor (818-ST2/DB) and Newport power meter 1936-R (Irvine, CA, USA). The red and near IR lights treatment was done at 0.3 J/cm² per day.

Human fibroblasts culture

Human adult dermal fibroblasts were purchased from Lifeline® Cell Technology (Frederick, MD). The cell culture materials were purchased from ThermoFisher Scientific (Bridgewater, NJ, USA), unless specified. The cells were seeded in phenol-red-free Dulbecco's modified Eagle's medium (PRF-DMEM) with 10% and 5% fetal bovine serum (FBS) for three days for collagen and elastin, respectively. Cells were then treated with red and near IR lights in the presence of 1X phenol-red free Hank's buffered saline solution (HBSS) per day for three days. The cells were re-incubated in PRF-DMEM with 0% and 1% FBS after light treatments for 72 hours for collagen and elastin, respectively. The cells were harvested for RNA analyses or were washed twice with cold 1x PBS and then lysed in cold lysis buffer (0.1 M Tris, 0.15 M NaCl, 0.5% Triton X-100) for ELISA or protein assays. Procollagen type I C-peptide (Takara Bio USA, Inc., Mountain View, CA, USA) enzyme-linked immunosorbent assay (ELISA) or elastin ELISA (Novatein Biosciences Inc., Woburn, MA, USA) and micro bicinchoninic acid (BCA) assay were done by following manufacturers' instructions. The ELISA results were expressed by total collagen or elastin content normalized by total protein content.

Human skin explants

Abdominal skin samples were obtained from normal human adults undergoing elective abdominoplasty surgeries with informed consents. Skin punch biopsies (10 mm) were prepared and acclimated in PRF-DMEM/F12 (1:1) medium, 2% heat-inactivated FBS, 10 µg/ml insulin, 10 ng/ml hydrocortisone (MilliporeSigma, St. Louis, MO, USA), 10 ng/ml epidermal growth factor, 1X antibiotics antimycotics (growth medium) [2625] overnight. Using this culture method, we have previously established that human skin explants are viable and biologically responsive for seven days. Skin explants were exposed to red and near IR lights' treatment in the presence of 1X HBSS and then returned to growth medium. The fresh medium was replaced every other day. The daily light treatments were done four days for gene expression analysis or for five days for histological examination.

Gene expression analysis

Total RNAs were isolated from human dermal fibroblasts or human skin biopsies using Qiagen RNeasy kit (Waltham, MA, USA) following the manufacturer's instructions. RNA concentration was assessed using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Bridgewater, NJ, USA).

Reverse transcription was performed using a High Capacity cDNA kit (ThermoFisher Scientific, Bridgewater, NJ, USA). TaqMan® gene expression assays for lysyl oxidase like protein 1 (LOXL1), elastin (ELN), alpha-1 type I collagen (COL1A1), alpha-1 type III collagen (COL3A1), polymerase (RNA) II polypeptide A (POLR2A) and TaqMan® gene expression master mix were purchased from ThermoFisher Scientific (Bridgewater, NJ, USA). Real time quantitative polymerase chain reaction (qPCR) was performed using a QuantStudio™ 7 Flex System (ThermoFisher Scientific, Bridgewater, NJ, USA). The RQ (relative quantitation) was calculated by using the formula $2^{-\Delta\Delta Ct}$ against POLR2A and the fold induction were further calculated in comparison to the untreated control.

Histochemical and immunohistochemical stains

Tissue sections (5 µm-thick) of skin explants were stained by Herovici stain for collagens [26,27,28] or Luna stain for elastic fibers without nuclear counterstain (American HistoLabs Inc, Gaithersburg, MD, USA). Images at 25X magnification were captured by a Leica DFC320 color digital camera and a Leitz Diaplan microscope (both from Leica Microsystems, Wetzlar, Germany) using ImagePro Plus (Media Cybernetics, Inc., Rockville, MD). Image analyses were performed using ImagePro Plus. Elastic fibers were analyzed by the % total area. For collagen quantitation, the blue or red channel was extracted and the mean intensity (MI) of blue or red color was recorded from the histogram for each image. The absolute intensity was calculated by subtracting the MI obtained from 255 (=255-MI) for each image. Collagen contents were expressed as the average absolute intensities. Immunohistochemical (IHC) staining were done by HistoWiz (Brooklyn, NY, USA) for collagen type I (1:400 ab34710, Abcam, Cambridge, United Kingdom) and type III (1:600 NBP105119, Novus Biologicals LLC, Centennial CO, USA). Images at 20X were captured. The MI and absolute intensity were obtained for each image. IHC of collagen contents were expressed as percent of untreated control.

Fluorescence emission spectroscopy

Serial fluorescence emission spectra of skin explants were measured at baseline (day 0) and at the end of experiment (day 5). The instrument used was a fiberoptic-based fluorescence spectrophotometer (SkinSkan, Horiba Jovin Yvon, Edison, NJ) equipped with a bifurcated fiber bundle 2 mm in diameter, consisting of 200 µm core diameter fibers, randomly mixed. Excitation was set to 330 nm or 450 nm with 5 nm bandwidth and emission intensity was scanned within 380 – 450 nm or 470 – 570 nm with 2 nm increments. The tip of the fiber bundle was placed against the skin in soft contact, with minimal pressure. Emission intensity maxima at 380 nm and 510 nm were used to assess collagen and elastic fiber crosslinks, respectively [28-3029-31]. Difference of intensities between day 0 and day 5 for each treatment was calculated, and further normalized by that of the untreated control.

ATP Luminescent Assay

ATP production was determined in human dermal fibroblasts at 5 minutes, 2 hours or 24 hours post-light treatments by the CellTiter-Glo® Luminescent cell viability assay (Promega, Madison, WI, USA) following manufacturer's instruction. Luminescence was read on the Promega GloMax Explore plate reader. The cell numbers were

quantitated by CellTiter-FluorTM cell viability assay (Promega, Madison, WI, USA) with Tecan Infinite M1000 plate reader (Tecan, Männedorf, Switzerland) following manufacturer's instruction. Normalized ATP values were obtained by total luminescence units divided by total cells' fluorescence.

Statistics

Statistical analyses were performed using two-tailed Student t-test (Microsoft Office Excel 2007, Microsoft, Redmond, WA, USA). Data were expressed as mean \pm standard deviation (SD). Differences were considered statistically significant if $p < 0.05$ (*).

Results

Red Plus Near IR LED lights Combination induced ELN, COL1A1 and COL3A1 gene expressions as well as elastin and procollagen production in human dermal fibroblasts in vitro

Red plus near IR lights combination induced significant ($p<0.05$) increases of ELN, COL1A1 and COL3A1 gene expressions (2.3 ± 0.6 fold, 1.4 ± 0.2 and 1.7 ± 0.2 fold, respectively) in human dermal fibroblasts as compared to that of untreated control (Fig. 1a). Red and near IR lights were found to induce significant ($p<0.05$) increases of elastin (169.7 ± 19.3 pg/ μ g protein vs 90.6 ± 41.2 pg/ μ g protein of untreated control) and procollagen type I (5.6 ± 0.9 vs 3.6 ± 1.0 ng/ μ g protein of untreated control) productions in human dermal fibroblasts (Fig. 1b and 1c).

Red Plus Near IR LED lights Combination induced elastin gene expression and elastic fiber formation in human skin ex vivo

Red plus near IR lights combination induced a significant ($p<0.05$) increase of elastin gene expression by 1.6 ± 0.7 fold as compared to that of untreated control in human skin explants (Fig. 2a). The representative images of elastin staining for the untreated and the red and near IR-treated skin tissues at day 5 are shown in Fig. 2b. The elastic fibers were in filamentous patterns in the DEJ and the upper papillary dermis (Fig. 2 b, arrows). Their corresponding quantitation of elastic fibers is shown in Fig. 2c and the light treatment induced significantly ($p<0.05$) more elastic fibers than that of untreated control ($0.14\% \pm 0.05\%$ vs $0.10\% \pm 0.02\%$ of total area), suggesting red and near IR lights enhanced elastic fiber production and assembly in human skin explants.

Red Plus Near IR LED lights Combination induced collagen gene expression and collagen production in human skin ex vivo

Red plus near IR lights combination induced significant ($p<0.05$) increases of COL1A1 and COL3A1 gene expressions (1.8 ± 0.7 and 1.6 ± 0.5 fold, respectively) as compared to that of untreated control in human skin explants (Fig. 3a). The representative images of collagen staining for the untreated and the red-and-near- IR-light-treated skin tissues at day 5 are shown in Fig. 3b. Young (type III) collagen was stained in blue color in the extracellular space of DEJ and the upper papillary dermis (Fig. 3b, arrows) whereas mature (type I) collagen was stained in red. Human skin explants treated with red and near IR lights showed significantly ($p<0.05$) more intensity of blue staining (124 ± 13 vs 103 ± 13 of untreated skin) and no significance in red staining (98 ± 18 vs 102 ± 12 of untreated skin) in the papillary dermis than that of the untreated skin explants (Fig. 3c).

Immunohistochemical staining of collagen type I (Fig. 4a) and type III (Fig. 4b) were done on the untreated and red-and-near-IR-light-treated skin explant tissue. The quantitation of the staining intensities showed both collagen type I ($112\% \pm 20\%$) and type III ($125\% \pm 28\%$) were significantly ($p<0.05$) higher than the untreated control (Fig. 4c).

Red Plus Near IR LED lights Combination induced LOXL1 gene and elastic fiber crosslinks in human skin explants ex vivo

Red plus near IR lights combination treatment significantly ($p<0.05$) induced LOXL1 gene expression (1.5 ± 0.3 fold) as compared to that of untreated control in human skin fibroblasts and also significantly ($p<0.05$) induced LOXL1 gene (1.6 ± 0.4 fold) in human skin explants as compared to that of untreated control (Fig. 5a). Red and near IR lights induced a significant ($p<0.05$) increase of elastic crosslinks (2.18 ± 0.79 fold) in human skin explants as determined by the fluorescence intensity at emission 510 nm than that of the untreated control (Fig. 5b). However, there was no change in collagen crosslinks in human skin explants by red and near IR lights' treatment as determined by the fluorescence intensity at emission 380 nm as compared to that of the untreated control (Data not shown).

Red Plus Near IR LED lights Combination induced ATP production in human dermal fibroblasts in vitro

Red plus near IR lights combination treatment significantly ($p<0.05$) increased ATP production after 5 minutes (291 ± 8 vs 250 ± 24 of untreated control) and 2 hours (167 ± 9 vs 142 ± 6 of untreated control) post-light treatments but not for 24 hours post-light treatment (Fig. 6).

Discussion

This study showed that the combination of red and near IR LED lights may significantly induce COL1A1, ELN, collagen and elastin protein formation in both human adult dermal fibroblasts and in human skin explants. Furthermore, it is shown for the first time that red plus near IR lights combination may also induce LOXL1 gene expression and enhance elastic fiber crosslinks, which are necessary for functional elastic fibers to contribute to skin structural support as skin firmness decreased with photo-aging. The mechanism of action (MOA) of red plus near IR lights combination treatment may be through inducing ATP production. Taken together, these results suggest that low-level red plus near IR lights combination can induce significant collagen and elastin production *in vitro* at energy levels of lights that are substantially lower than those used clinically.

Red or near IR light may have beneficial effects on wound healing by inducing collagen synthesis [12,16] or decreasing MMP-1 [9]. Weiss *et al* [25] showed clinical use of the low intensity (0.1 J/cm^2) 590 nm wavelength LED array pulsed light is highly effective for stimulation of collagen synthesis and for production of clinical improvement in photoaging. In this study, red plus near IR lights combination induced procollagen type I production in human dermal fibroblasts (Fig. 1 c), increased type III collagen by Herovici stain in skin explants (Fig. 3 b, 3c) [3432] and increased collagen type I and type III by IHC staining in the DEJ and upper papillary dermis of human skin explants (Fig. 4a-4c). Comparing results from this study with the results of Weiss *et al* [25], suggests that the increase in collagen and ECM protein expression would result in a clinical benefit for photo-damaged skin. Weiss *et al* [25] reported an 28% increase in collagen protein staining in LED light treated skin, which is comparable to the 25% increase in collagen staining we observed (Fig. 4) using low level (0.3 J/cm^2) red plus near IR lights combination. Taken together with Weiss *et al* [25], these results suggest that the red plus near IR light combination does induce photo-biomodulation by activating the matrix genes to build more extracellular matrix which would lead to clinical improvement in photoaging.

Enzymes LOX and LOXL1 are known to initiate the crosslinking of collagen and elastin by catalyzing the formation of the lysine-derived aldehyde [3233]. LOX was found only in differentiating keratinocytes whereas LOXL1 was found in keratinocyte, proliferating and differentiating fibroblasts [3334]. LOX is responsible for the fibrillar collagens crosslinking and plays an important role in life as LOX-deficiency was lethal [34-3635-37]. LOXL1 plays a dual role in elastic fiber formation and in their renewal but does not involve in collagen crosslinking [33,3734,38]. Fluorescence excitation spectroscopy has been used to detect specific autofluorescent molecules such as collagen and elastin crosslinks in skin [28,29,30]. Lutz *et al* [3839] showed that collagen fluorescence reduction was corresponding to reduced collagen crosslinking. In this study, red plus near IR lights combination induced LOXL1 gene expression (Fig. 5a) and increased fluorescent intensity of elastic fiber crosslinks in human skin explants (Fig. 5b). There was no increase fluorescence of collagen crosslinks by red plus near IR lights combination treatment (Data not shown). Although UV may induce elastin mRNA and protein production in skin, the elastin was

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accumulated as a large clump of fibers in the papillary dermis [3940]. In addition, UV couldn't induce LOXL1 protein and its enzyme activity in the photoaged skin [4041]. In this study, red plus near IR lights combination induced elastic fiber formation in human skin explants (Fig. 2 b), induced LOXL1 gene (Fig. 5a) and increased elastin crosslinks in human skin explants (Fig. 5 b) that may lead to functional elastic fiber structure instead.

Previous studies have suggested cytochromes c oxidase as one of the primary photo-acceptors absorbing red and near IR lights. Irradiation at 660 nm might influence cytochrome c oxidase that leads to increased mitochondrial activity and ATP synthesis [7]. Low-level LED therapy (2.5 J/cm^2) of red (630 nm) and near IR (830 nm) induced significant ATP production 3- and 6-hour-post-light treatments in C2C12 mouse muscle cells [8]. Changes in mitochondrial respiratory chain modulated signaling molecules to stimulate synthesis of RNA and proteins and result in induced cell proliferation and differentiation [41,42,43]. This study showed that ATP production was increased up to 2 hours post-light treatment in human fibroblasts when treated with low-level red plus near IR lights combination (Fig. 6). Through this ATP increase, the gene and protein expressions of collagen and elastin were increased after low-level red plus near IR lights combination treatment.

In conclusion, this study demonstrated that low-level dual-wavelength red plus near IR lights combination could induce collagen and elastin syntheses in human fibroblasts *in vitro* and in human skin explants *ex vivo*. This combination of red and near IR lights also induced LOXL1 gene expression and enhanced elastic fiber crosslinks in human skin *ex vivo*. By increasing collagen and elastin production, and by increasing the expression of LOXL1, low-level red plus near IR lights combination may improve the loss of skin firmness, sagging, and fine lines/wrinkles in photo-damaged and aging skin. Taken together, these results suggest that low-level red plus near IR lights combination may provide an effective treatment opportunity for people with photo-aged skin.

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Legends

Figure 1 Red and near IR lights (each at 0.3 J/cm²) induced ELN, COL1A1 and COL3A1 gene expressions, procollagen and elastin production in human adult fibroblasts after three days' treatment. (a) Red and near IR lights effect on ELN, COL1A1 and COL3A1 gene expression in human dermal fibroblasts. qPCR analysis was used to evaluate the gene expression. Results were represented as mean ± SD, n=4. (b) Red and near IR lights effect on elastin protein production in human dermal fibroblasts. Elastin protein in the cells was measured by ELISA and normalized by total protein. Total protein content was measured by MicroBCA assay. Results were represented as mean ± SD, n=5. (c) Red and near IR lights effect on procollagen type I protein production in human dermal fibroblasts. Procollagen type I protein in the cells was measured by ELISA and normalized by total protein. Total protein content was measured by MicroBCA assay. Results were represented as mean ± SD, n=6. UT, untreated; R+NIR, red and near IR lights; *, p<0.05 vs UT.

Figure 2 Red and near IR lights (each at 0.3 J/cm² per day) increased elastin gene and protein production in human skin explants after up to five days' treatments. (a) Red and near IR lights effect on elastin gene expression in human skin explants after four days' treatment. qPCR analysis was used to evaluate the gene expression. Results were represented as mean ± SD, n=8. (b) Representative histological images of elastic fibers (arrows) in human skin explants after five days' treatment. Elastic fibers were stained by Lunar stain, which were shown in purple color. (c) Quantitation of elastic fibers in human skin explants after five days' treatment. The % area of the elastic fiber in dermal-epidermal junction and the upper papillary dermis was measured by ImagePro Plus. Results were represented as mean ± SD, n=36. UT, untreated; R+NIR, red and near IR lights; Original magnification, 250X; *, p<0.05 vs UT.

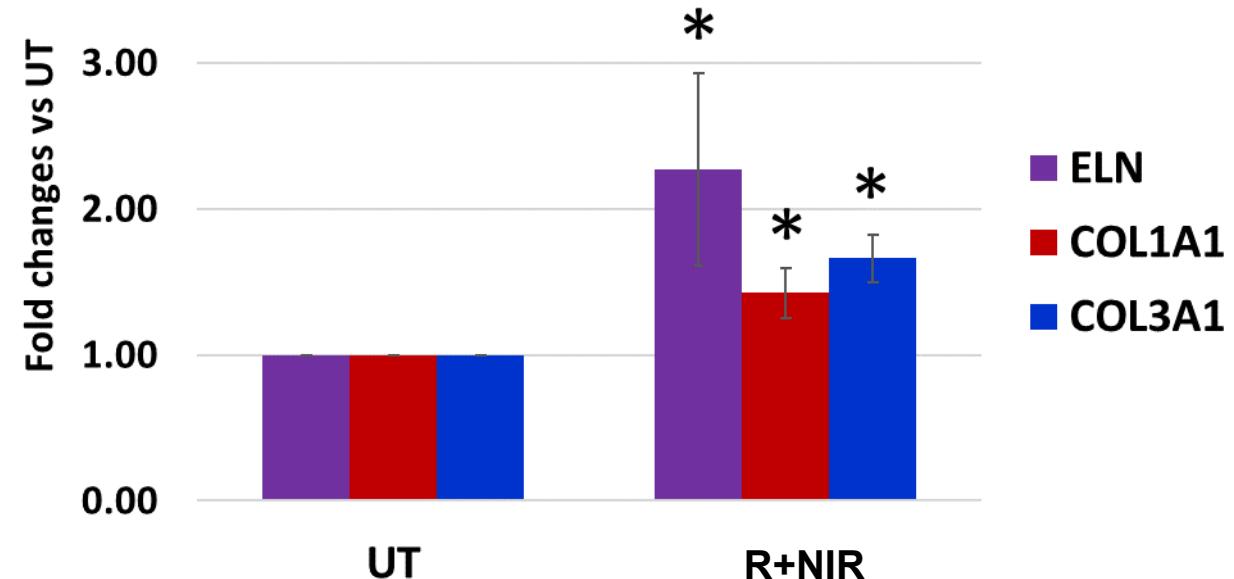
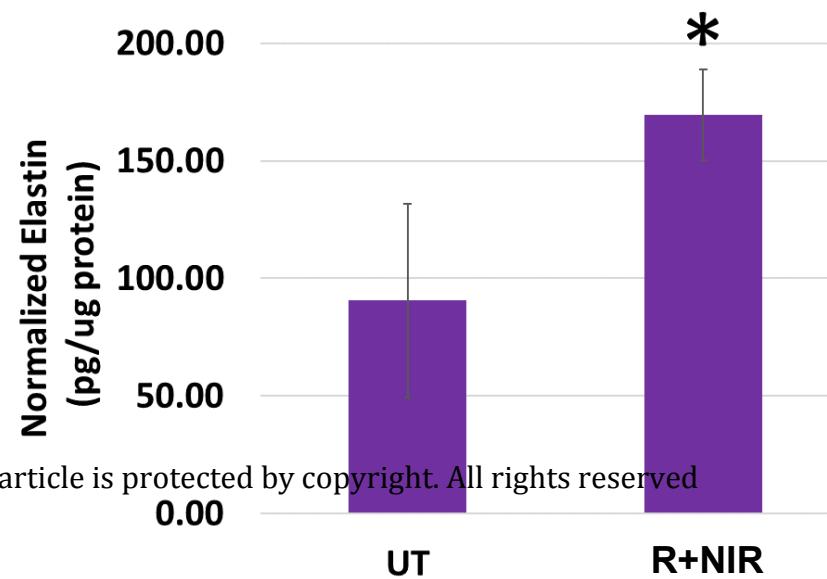
Figure 3 Red and near IR lights (each at 0.3 J/cm² per day) increased COL1A1 and COL3A1 genes and collagen production in human skin explants after four to five days' treatment. (a) Red and near IR lights effect on COL1A1 and COL3A1 gene expressions in human skin explants after four days' treatment. qPCR analysis was used to evaluate the gene changes. Results were represented as mean ± SD, n=8. (b) Representative histological images of young collagen type III (blue color, arrows) and mature collagen type I (red color) in human skin explants after five days' treatment. Collagens were stained by Herovici stain. (c) Quantitation of staining intensities of collagen type III (blue) and mature collagen type I (red) in human skin explants after five days' treatment. The red or blue color channels was extracted from each image and the mean intensities (MI) of blue- and red-stained collagens of the whole image were measured by ImagePro Plus. The absolute intensities were adjusted by subtracting the MI obtained from 255 (=255-MI). Results were represented as mean ± SD, n=27. UT, untreated; R+NIR, red and near IR lights; Original magnification, 250X; *, p<0.05 vs UT.

Figure 4 (a) Representative images of immunostaining of collagen type I in human skin explants after five days' treatment. Collage type I (brown color) was present in DEJ regions and dermis. Bar, 100 μm. (b) Representative images of immunostaining of collagen type III in human skin explants five days' treatment. Collagen type III (in

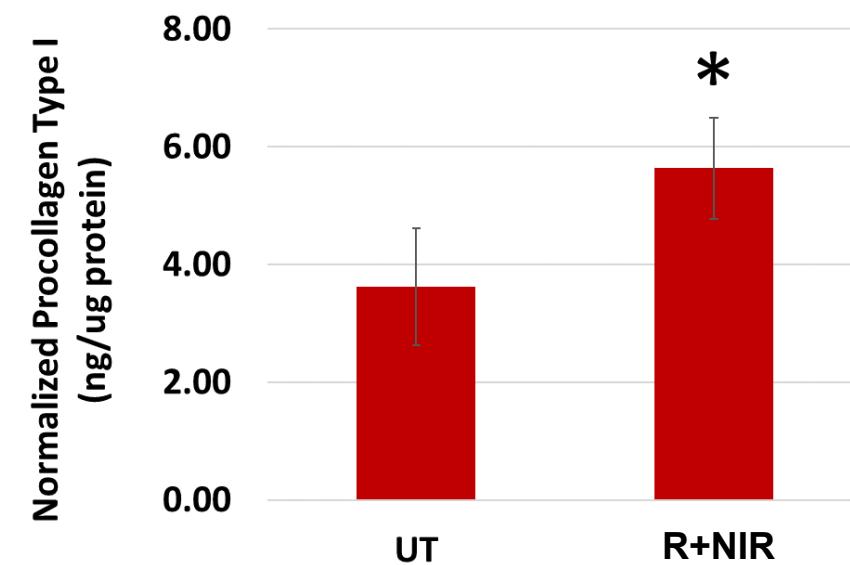
brown color) was in DEJ regions and dermis as well. Bar, 100 μ m. (c) Quantitation of immunostaining intensities of collagen type I and type III after five days' treatment. The staining intensity was expressed as percent of untreated control. Results were represented as mean \pm SD, n=27. UT, untreated; R+NIR, red and near IR lights; *, p<0.05 vs UT.

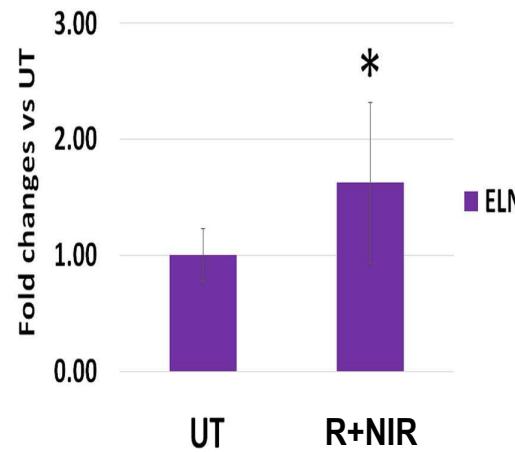
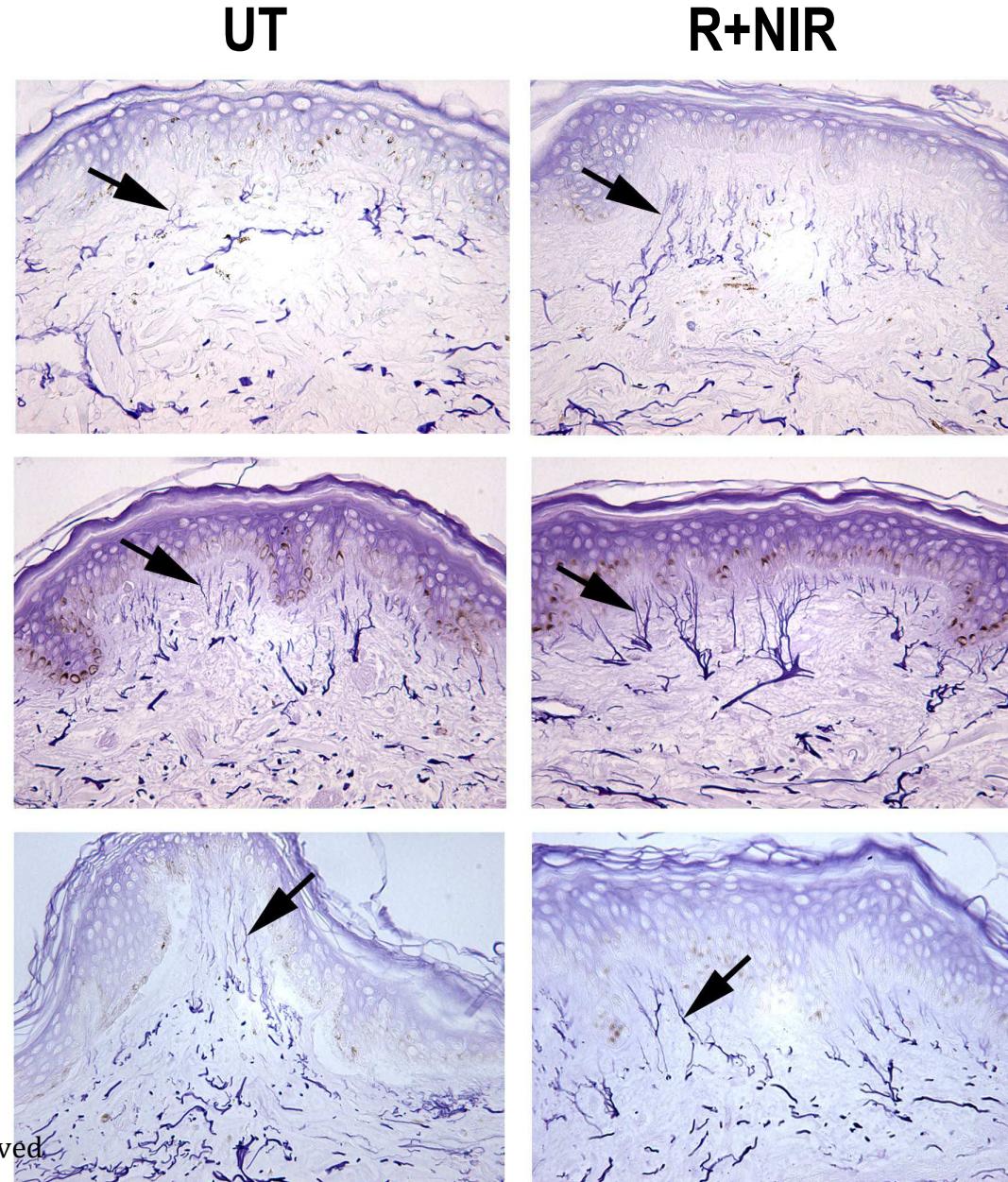
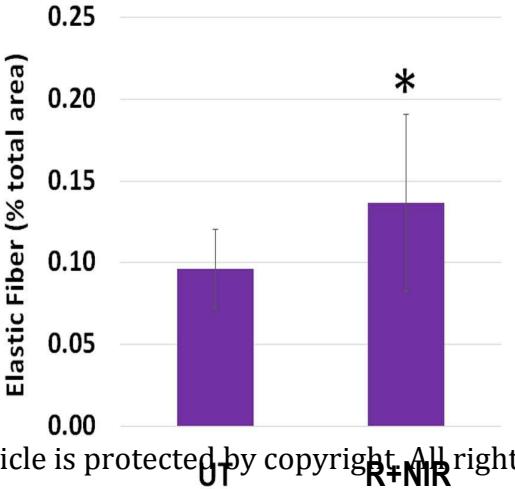
Figure 5 Red and near IR lights (each at 0.3 J/cm² per day) increased LOXL1 gene expression in human dermal fibroblasts and skin explants as well as elastin crosslinks in human skin explants. (a) Red and near IR lights effect on LOXL1 gene expression in human dermal fibroblasts for three days' treatment and skin explants for four days' treatment. qPCR analysis was used to evaluate the gene expression. Results are represented as mean \pm SD, n=6. (b) Red and near IR lights effect on elastic fiber crosslinks. Elastic fiber crosslinks in human skin explants were measured by fluorescence emission spectroscopy to detect the skin fluorescence intensity at emission 510 nm on day 0 and day 5. The change of fluorescence intensity on day 5 was calculated using the intensity on day 5 minus the intensity on day 0. The elastin crosslinks were expressed as % of UT using the increased fluorescence units at day 5 normalized by that of untreated control. Results were represented as mean \pm SD, N=4. UT, untreated; R+NIR, red and near IR lights; *, p<0.05 vs UT.

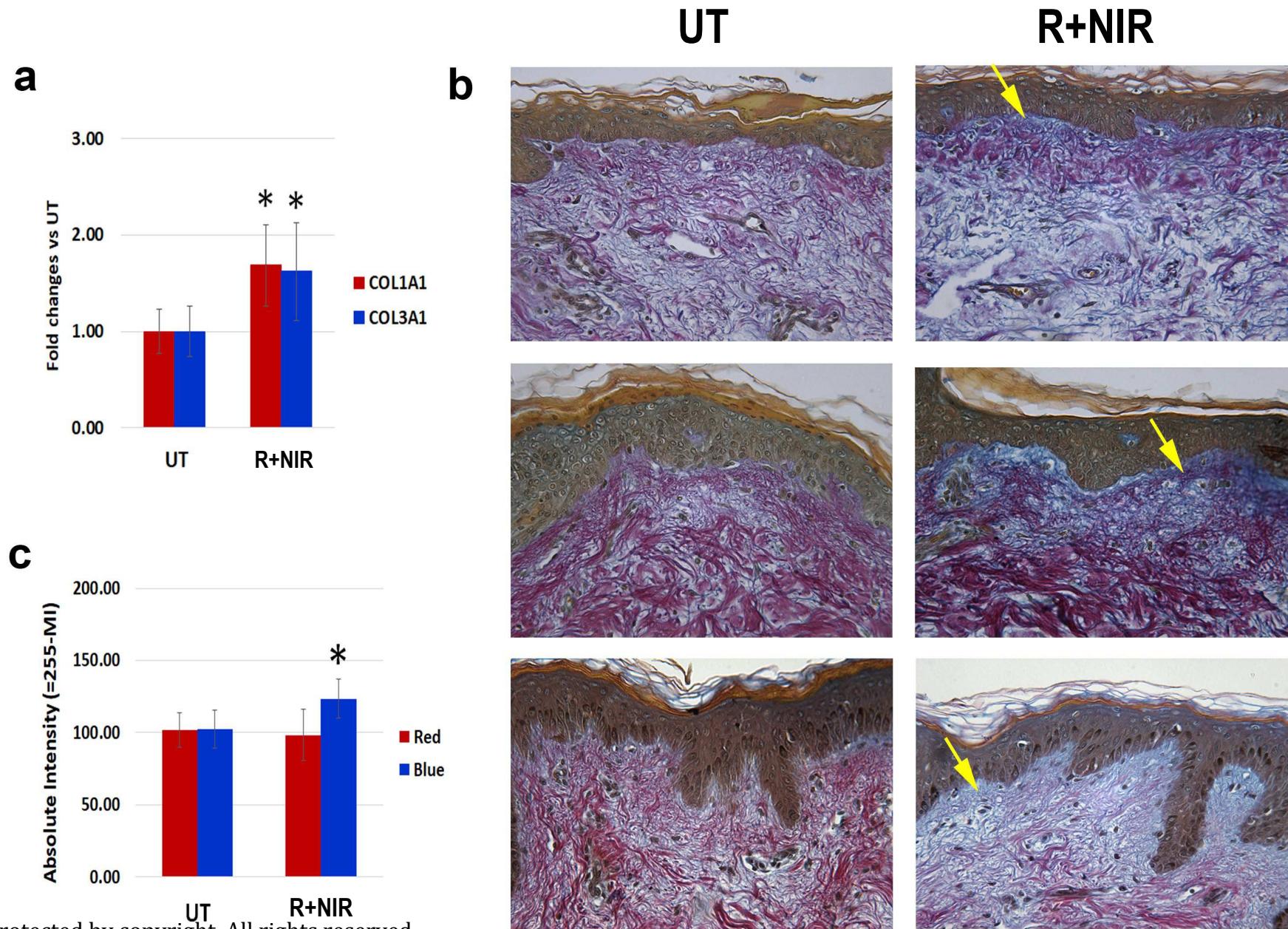
Figure 6 Red and near IR lights effect on ATP production. Red and near IR lights (each at 0.3 J/cm²) induced ATP production 5 minutes and 2 hours post-light treatments in human dermal fibroblasts. ATP was measured after 5 minutes, 2 hours or 24 hours post-light treatments in human dermal fibroblasts. CellTiter-Glo® ATP luminescent assay was used to measure ATP production and CellTiter-Fluor™ assay was used to measure cell numbers. ATP was normalized by total cells' fluorescence as shown in arbitrary units. Results were represented as mean \pm SD, n=6. UT, untreated; R+NIR, red and near IR lights; *, p<0.05 vs UT.

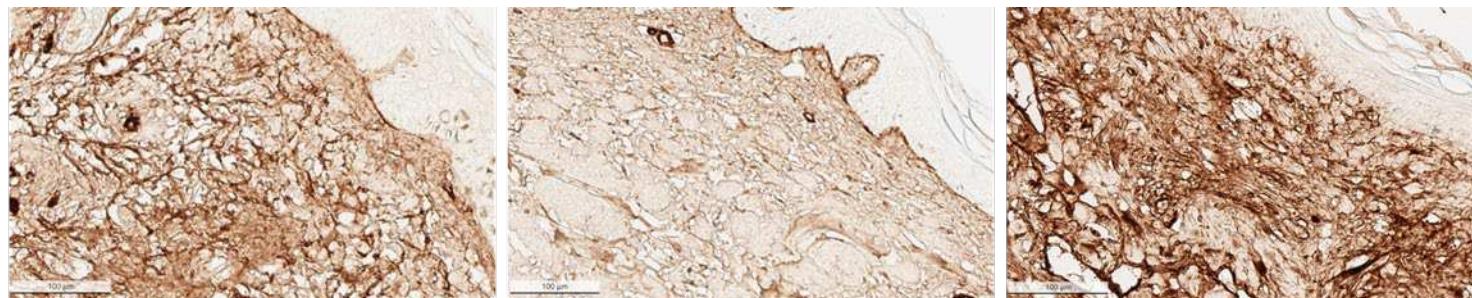
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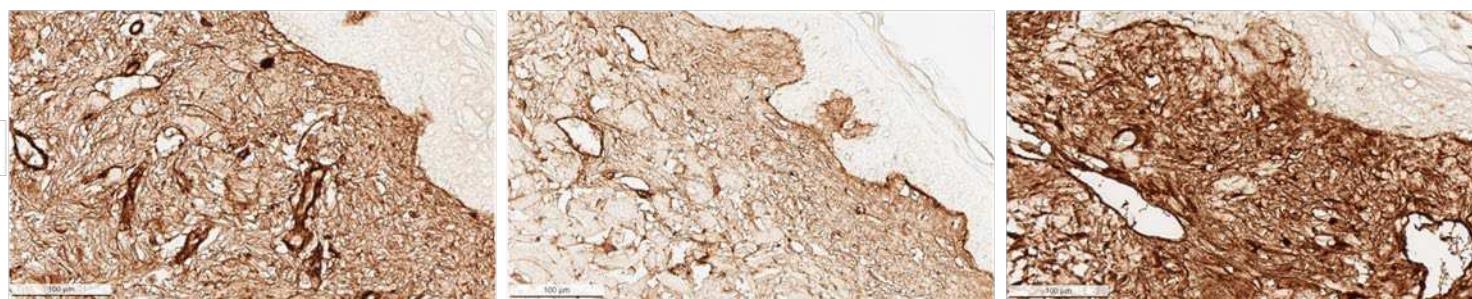
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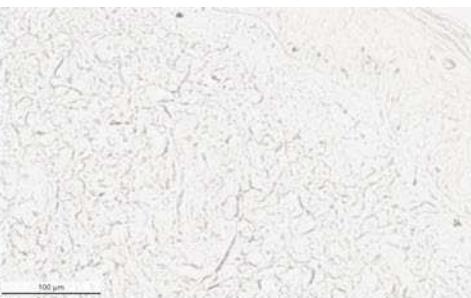
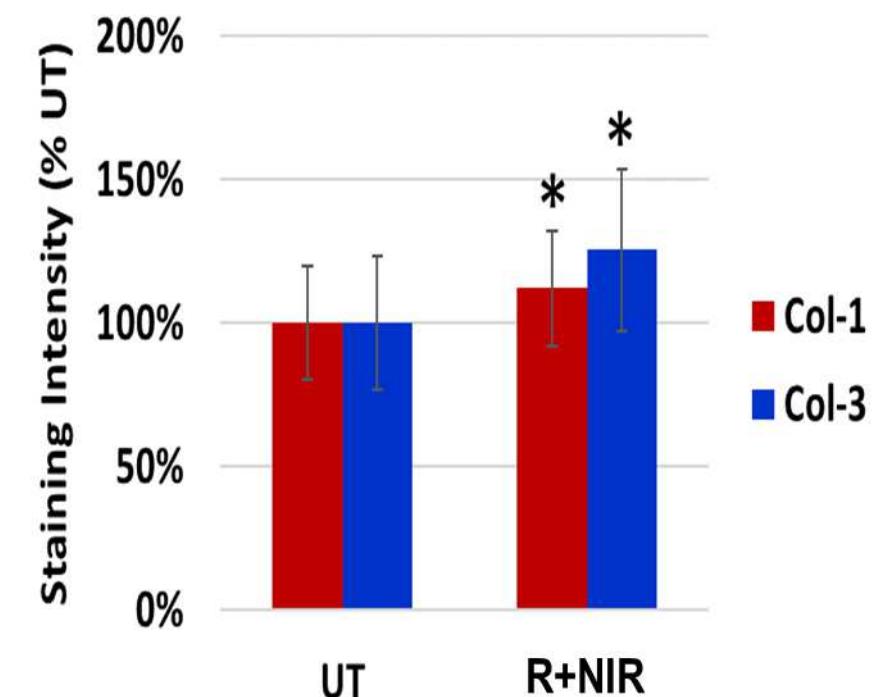


a

UT

b

R+NIR

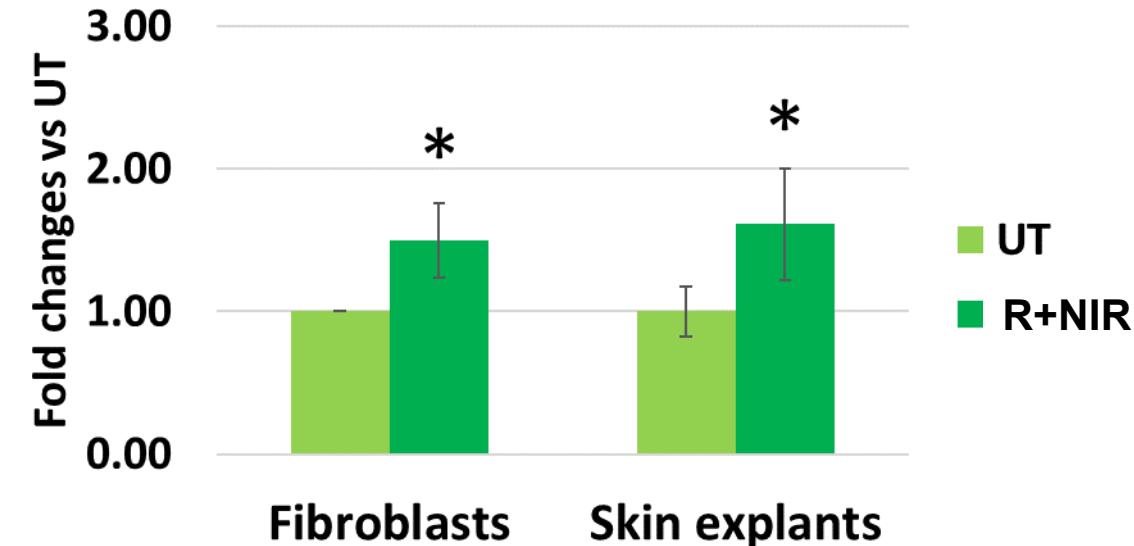
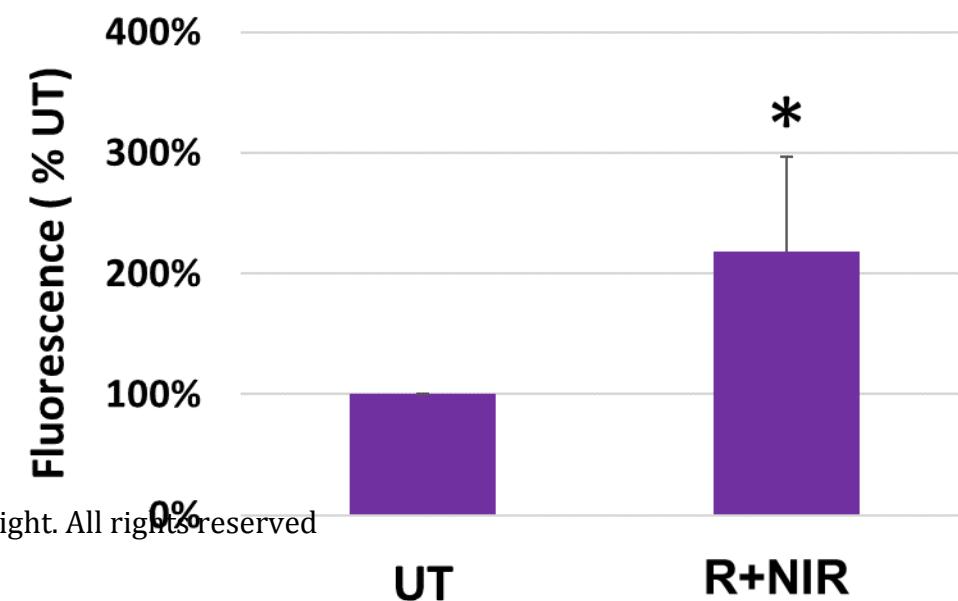
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UT



R+NIR

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a**b****Elastic Fiber Crosslinks**

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ATP Production