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Cutaneous Photorejuvenation of Light Emitting Diodes via the Melatonin Membrane Receptor Pathway

Soo Hyeon Bae, Jun Hyeong Park, Soo-Hyun Kim, Sook Jung Yun, Jae Gwan Kim[1], Jee-Bum Lee

Department of Dermatology, Chonnam National University Medical School, Gwangju, [1]Department of Biomedical Science and Engineering, Gwangju Institute of Science and Technology, Gwangju, Korea

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Corresponding Author
Jee-Bum Lee
Department of Dermatology, Chonnam National University Medical School, 42 Jebong-ro, Dong-gu, Gwangju 61469, Korea
Tel: +82-62-220-6682
Fax: +82-62-220-6685
E-mail: jbmlee@jnu.ac.kr
https://orcid.org/0000-0002-1477-4037

Background: Melatonin receptors are present in the human skin and retina. These receptors can be stimulated by light emitting diodes (LEDs) at specific wavelengths, thereby inducing cutaneous photorejuvenation. However, the underlying mechanism remains unclear.
Objective: To evaluate the influence of LEDs at specific wavelengths on melatonin membrane receptor (MT1) and cutaneous photorejuvenation via the MT1 pathway in vitro.
Methods: Normal human dermal fibroblasts (HDFs) were irradiated using LEDs at different wavelengths (410~940 nm) at a dose of 1 J/cm^2. MT1 activity was evaluated after melatonin stimulation and LED irradiation. Thereafter, the expressions of collagen (COL) and matrix metalloproteinases (MMPs), with and without luzindole (MT1/2 receptor antagonist), were investigated via semi-quantitative reverse transcription polymerase chain reaction (PCR), real-time PCR, western blotting, and enzyme-linked immunosorbent assay.
Results: In HDFs, the MT1 mRNA and protein levels increased significantly in response to melatonin (dose, 50 nM) (p<0.01) and LED irradiation at 595, 630, 850, and 940 nm (p<0.01). LED irradiation up-regulated COL type I and down-regulated MMP-1. Compared to LED irradiation without luzindole, LED irradiation with luzindole produced no significant increase in COL type I mRNA and protein levels (p<0.01).
Conclusion: We found that LED irradiation induces collagen synthesis and MMP-1 inhibition in HDFs via MT1 activation. Additionally, multiple LED wavelengths (595, 630, 850, and 940 nm) stimulated MT1 in HDFs, unlike in the eyes, where only blue light induced plasma melatonin suppression. This suggests the possibility of the melatoninergic pathway in photorejuvenation.

Keywords: Light emitting diode, Melatonin, Photorejuvenation

INTRODUCTION

Melatonin is a hormone long known for its chronobiological effects, including the regulation of daily circadian rhythm and seasonal bio-rhythms[1]. It also acts as a direct or indirect anti-oxidant[2,3], modulates immune defence responses[4], and has anti-cancer[5] and anti-jet-lag[6] effects. Interestingly, recent studies have focused on the cutaneous rejuvenative feature of this hormone[7], because melatonin can significantly reduce ultraviolet-B-induced cell damage and accelerate the wound healing process[8,9].

The pineal gland is the major melatonin-secreting organ; however, melatonin is also present in other organs, such as the skin, immune system, mammary gland, gastrointestinal tract, liver, kidney, urinary bladder, ovary, testis, and prostate[10,11]. The biological effect of melatonin is attributed to the activation of its membrane receptors[12]. Melatonin membrane receptors, MT1 and MT2, are expressed in humans and other mammals, while MT3 is present in amphibians and birds[13]. While MT1 and MT2 expression varies depending on the organs, human skin expresses a strong bias toward MT1[14].

Light is the primary trigger for melatonin production in the eye[15]; its production depends on the wavelength, duration, intensity, and timing of light. Blue light, especially in the wave-

length range of 446 to 477 nm, significantly suppresses plasma or salivary melatonin, while blue light at longer wavelengths shows lesser suppression[16-18]. Although human skin expresses melatonin receptors, the melatonin-based cutaneous response to light irradiation remains unrevealed. Previous studies have shown that different types of cells and species show different action spectra of melatonin in response to light[16,19]. Therefore, we assumed that human skin may show a peculiar melatoninergic response to light.

Photobiomodulation, also known as low-level light therapy (LLLT), was introduced approximately 50 years ago. Moreover, numerous studies have elucidated the cutaneous effects of light-emitting diodes (LED) on skin rejuvenation and wound healing[20-22]. Several possible mechanisms have been proposed to explain these effects, including cytochrome C oxidase and light-sensitive ion channel activation, which leads to the activation of transcription factors related to protein synthesis, cell migration and proliferation, anti-inflammatory signalling, and anti-oxidant enzymes[23]. As the cutaneous effects of melatonin, such as rejuvenation, anti-oxidation, and wound healing[2,24,25], are parallel to those of LLLT and melatonin is a well-known light-induced hormone, we also intended to evaluate the role of melatonin as a mediator of photorejuvenation.

In the present study, we aimed to investigate the influence of different wavelengths of LED light on skin melatonin activity by measuring melatonin receptor expression in LED-irradiated human dermal fibroblasts (HDFs) in vitro. We also investigated whether LED irradiation of HDFs at specific wavelengths showed skin rejuvenative effects in accordance with melatonin activity.

MATERIALS AND METHODS

The study was approved by the Institutional Review Board of the Chonnam National University Hospital (IRB no. CNUH-EXP-2022-320). The informed consent was waived.

LED light sources and reagents

LEDs at wavelengths of 940±2, 850±3, 630±8, 595±2, 580±4, 525±4, 480±7, and 410±10 nm were used for irradiation. Each LED irradiation dose was measured using a quantum photoradiometer (Delta OHM, Padova, Italy) connected with a visible probe (Sonda LP 9021 RAD; Delta OHM). LED with different wavelengths was applied with uniform fluences ranging from 1 to 5 J/cm^2. The distance from the LED module to the cells was 5 cm. Melatonin (M5250; Sigma, St. Louis, MO, USA) and luzindole (CAS 117946-91-5; Santa Cruz biotechnology, Inc., Santa Cruz, CA, USA) were used as the reagents.

Cell culture

Human Dermal Fibroblasts, neonatal were purchased from EpiLife (Cascade Biologics, Portland, OR, USA). Cell cultures were maintained in Dulbecco's Modified Eagle's Medium (Bio-Whittaker Cambrex Bio Sciences, Walkersville, MD, USA) supplemented with 10% foetal bovine serum and 2 mM glutamine.

Cell viability

Neonatal human dermal fibroblasts (6×10^3 cells/well) were seeded in a 96-well plate. After application of melatonin at various concentrations, cell viability was assayed using the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide kit (Chemicon International Inc., Billerica, MA, USA), according to the manufacturer's instructions.

Semi-quantitative reverse transcription PCR and real-time PCR

Total mRNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA, USA). Subsequently, cDNA was reverse transcribed from 500 ng of total RNA with the Omniscript RT kit (Qiagen) and subjected to semi-quantitative reverse transcription polymerase chain reaction (semi-qRT-PCR) with HiPi PCR PreMix (Elpis Biotech, Daejeon, Korea). Table 1 shows the primer sequences and product sizes. Semi-qRT-PCR products were analysed using 1.5% agarose gel electrophoresis, stained with Sybr Safe DNA gel stain buffer (Invitrogen, Carlsbad, CA, USA), and visualised using luminescence (LAS 3000; Fujifilm, Tokyo, Japan). Expression levels were normalised to those of the endogenous control GAPDH. To measure mRNA levels, RT-PCR and quantitative real-time PCR were performed with the same primer sets for target genes. Real-time PCR was performed in triplicate with the HOT FIREPol EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) using a RotorGene 3000 system (Corbett Research, Cambridge, UK). The thermal cycling conditions were as follows: 15 minutes at 95℃, followed by 50 cycles of 95℃ for 10 seconds, 55℃ for 20 seconds, and 72℃ for 30 seconds. The relative abundance of a given transcript was estimated using the $2^{-\Delta\Delta Ct}$ method, following normalisation to that of the endogenous control GAPDH.

Table 1. Primer sense and anti-sense sequences

Gene	Primer sequences (forward/reverse)	PCR product size (bp)
MT1 (melatonin receptor 1A)	5′-ttgtccttttgccatttgctg-3′/5′-gtcatcagtggagacggtttcc-3′	289
MT2 (melatonin receptor 1B)	5′-ctaccaccgaatctaccggc-3′/5′-gacacgacagcgatagggag-3′	210
COL-I (Type 1 collagen, α1)	5′-atgatgagaaatcaaccgga-3′/5′-ccagtagcaccatcatttcc-3′	487
COL-III (Type 3 collagen, α1)	5′-cctccaactgctcctactcg-3′/5′-tcgaagcctctgtgtccttt-3′	536
MMP-1	5′-agatgtggagtgcctgatgt-3′/5′-tgcaacacgatgtaagttgt-3′	378
TIMP-1	5′-acccccgccatggagagtgt-3′/5′-gaggcaggcaggcaaggtga-3′	319
TGF-β1	5′-gggactatccacctgcaaga-3′/5′-cggagctctgatgtgttgaa-3′	124
GAPDH	5′-gtcttcaccaccatggagaaggc-3′/5′-cggaaggccatgccagtgagctt-3′	400

MMP: matrix metalloproteinase, TIMP: tissue inhibitors of metalloproteinase, TGF: transforming growth factor.

Western blotting

Western blotting was performed as described previously[26]. Protein bands were probed with rabbit antibodies against anti-MEL-1A/B-R (B-8, sc-398788; Santa Cruz biotechnology, Inc.), anti-Pro-COL1A2 (Y-18, sc-8787; Santa Cruz biotechnology, Inc.), anti-matrix metalloproteinase (MMP)-1/8 (H-300, sc-30069; Santa Cruz biotechnology, Inc.), and anti-β-actin (ab-6276; Abcam, Cambridge, MA, UK) overnight at 4°C. Table 2 shows the antibodies used.

The protein bands were visualised using luminescence (LAS 3000; Fujifilm). Densitometric analyses were performed using Multi Gauge V3.0 software (Fujifilm). Expression levels were normalised to those of the endogenous control β-actin. Data are representative of the three experiments.

Enzyme-linked immunosorbent assay (ELISA)

Commercial enzyme-linked immunosorbent assay (ELISA) kits were used according to the manufacturers' protocols to quantify the immune molecules of interest: human MT-NR1A/Melatonin Receptor 1a (LSBio, Seattle, WA, USA). Pro-collagen I protein (PIP) levels were determined using a commercially available pro-collagen type I C peptide enzyme immunoassay kit (Takara, Shiga, Japan).

Statistical analysis

Values are expressed as means±standard deviations. Statistical analyses were performed via one-way analysis of variance with a post-hoc Scheffé's test using IBM SPSS Statistics for Windows/Macintosh, ver. 25.0 (IBM Corp., Armonk, NY, USA), when multiple comparisons were made. A p-value <0.05 was considered statistically significant.

Table 2. Antibodies

Antibody	Dilution
1st	
Anti-MEL-1A/B-R (B-8) (sc-398788; Santa Cruz biotechnology, Inc., Santa Cruz, CA, USA)	1:200
Anti-Pro-COL1A2 (Y-18) (sc-8787; Santa Cruz biotechnology, Inc.)	1:200
Anti-MMP1/8 (H-300) (sc-30069; Santa Cruz biotechnology, Inc.)	1:200
Anti-β-actin (ab-6276; Abcam, Cambridgeshire, UK)	1:5,000
2nd	
Goat anti-rabbit IgG-HRP (sc-2004; Santa Cruz biotechnology, Inc.)	1:5,000
Donkey anti-goat IgG-HRP (sc-2020; Santa Cruz biotechnology, Inc.)	1:5,000
Goat anti-mouse IgG-HRP (sc-2005; Santa Cruz biotechnology, Inc.)	1:5,000

RESULTS

Effects of melatonin and LED irradiation on cell survival

Cell viability was evaluated after melatonin treatment at various doses (Supplementary Fig. 1A). No effects were observed. Cell viability was also evaluated after LED irradiation at various doses (1, 2.5, 5, 10, and 20 J/cm^2) and wavelengths (940±2, 850±3, 630±8, 595±2, 580±4, 525±4, 480±7, and 410±10 nm). Almost no effects were observed; however, reduced cell viability was noted at a wavelength of 410±10 nm and a dose of ≥10 J/cm^2 (p<0.05 vs. control) and at a wavelength of 480±7 nm and a dose of 20 J/cm^2 (p<0.05 vs. control) (Supplementary Fig. 1B).

Application of melatonin and LED irradiation on melatonin receptor expression in HDFs

Significant increases in the mRNA levels of MT1 were observed after melatonin stimulation of HDFs at doses ≥50 nM (Fig. 1A, B). Western blot analysis showed that melatonin receptor expression also significantly increased after melatonin stimulation at doses ≥ 25 nM (Fig. 1C).

When LED irradiation was applied to HDFs, the mRNA levels of MT1 significantly increased at the wavelengths 940±2, 850±3, 630±8, 595±2, 580±4, 525±4, 480±7, and 410±10 nm at a dose of 1 J/cm^2 (Fig. 1D, E). Among these LED wavelengths (dose, 1 J/cm^2), 940±2, 850±3, 630±8, and 595±2 nm showed more significant MT1 activity (p<0.01 vs. control). On western blot analysis, melatonin receptor expression in HDFs significantly increased with LED irradiation at the wavelengths 940±2, 850±3, 630±8, 595±2, 580±4, 525±4, 480±7, and 410±10 nm at a dose of 1 J/cm^2 (Fig. 1F).

Regulation of collagen and MMPs after melatonin stimulation and LED irradiation of HDFs

The mRNA levels of COL-I (type I collagen α1) and COL-III significantly increased after melatonin stimulation at doses ≥50 nM in HDFs (Fig. 2A). The mRNA levels of transforming growth factor-β1 (TGF-β1) increased while those of MMP-1 decreased after melatonin stimulation at doses ≥50 nM in HDFs (p<0.01) (Fig. 2B). On western blot analysis and ELISA, a significant increase in the expression of type I procollagen and a decrease in that of MMP-1 were also observed after melatonin stimulation at doses ≥25 nM (p<0.01) (Fig. 2C, D).

On LED irradiation of HDFs, the mRNA levels of COL-I

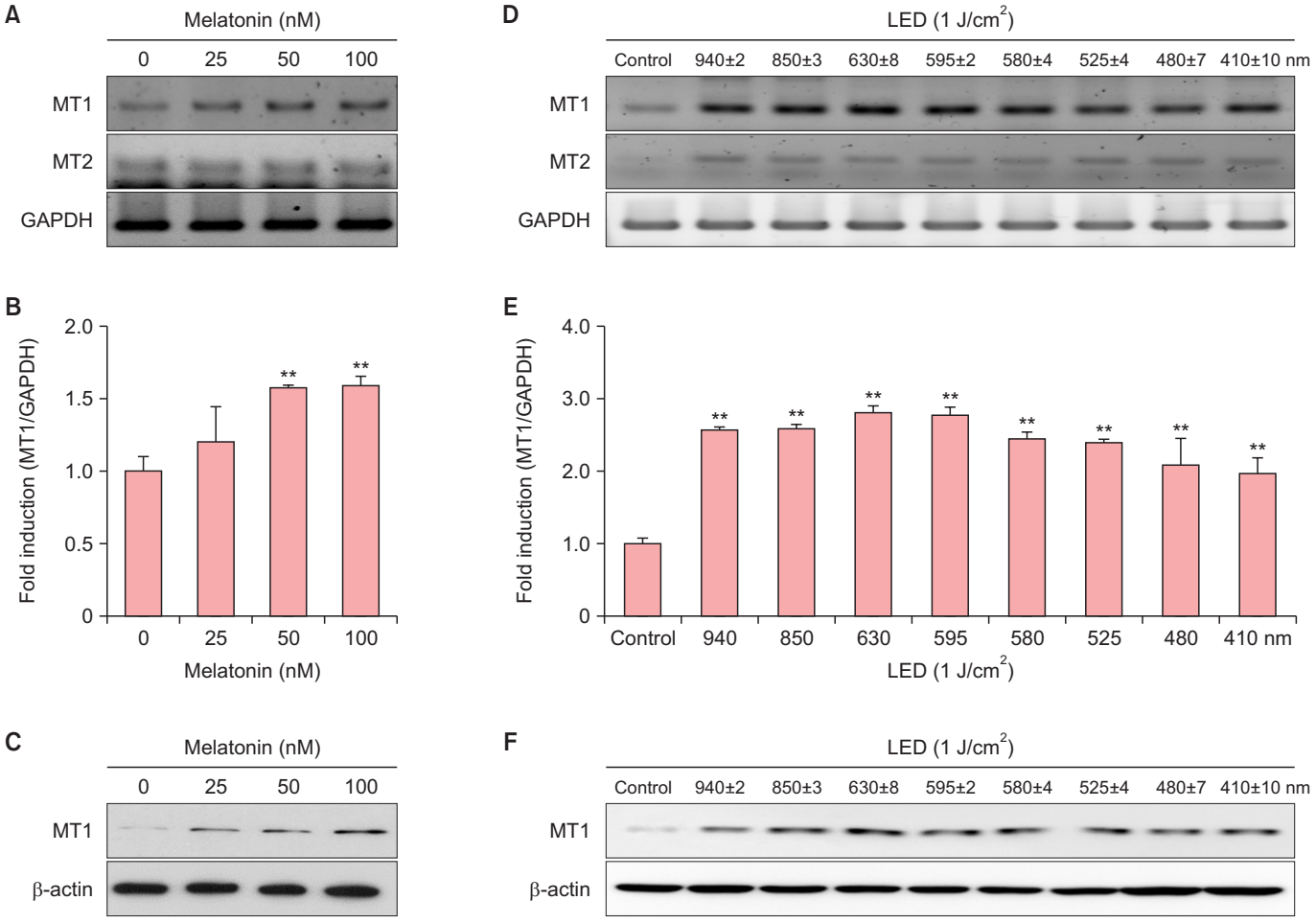

Fig. 1. Melatonin receptors identified in human dermal fibroblasts (HDFs). The impact of melatonin stimulation on mRNA and protein levels of melatonin receptors in HDFs; (A) semi-qRT-PCR, (B) real-time PCR, and (C) western blotting. The impact of various light emitting diodes (LEDs) at wavelengths (dose: 1 J/cm^2) of 940±2, 850±3, 630±8, 595±2, 580±4, 525±4, 480±7, and 410±10 nm on mRNA and protein levels of melatonin receptors in HDFs; (D) semi-qRT-PCR, (E) real-time PCR, and (F) western blotting. **p< 0.01 vs. normal control.

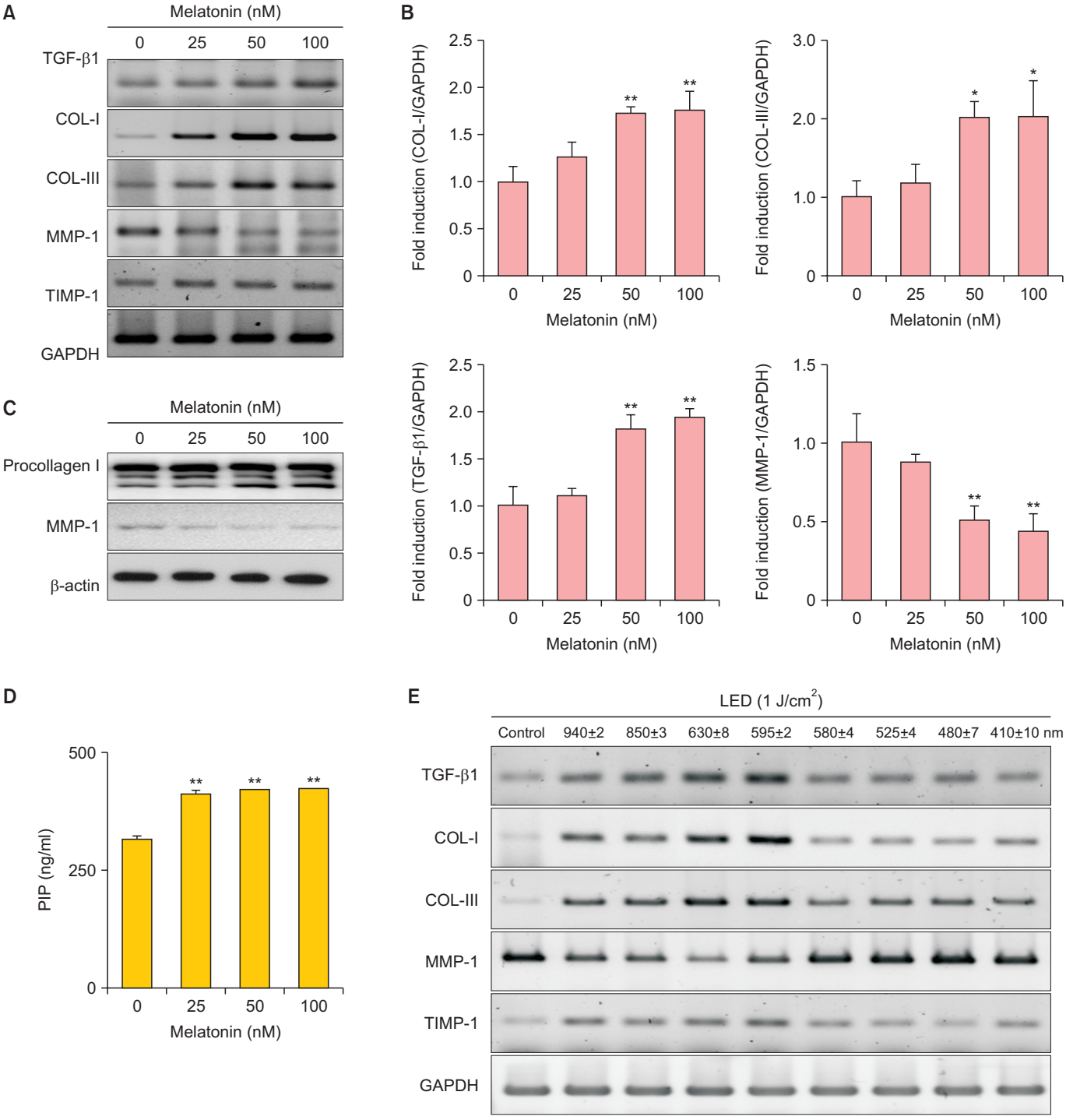

Fig. 2. Regulation of collagen synthesis identified in human dermal fibroblasts (HDFs). The impact of melatonin stimulation on mRNA and protein levels of transforming growth factor-β1 (TGF-β1), collagen (COL)-I, COL-III, matrix metalloproteinase (MMP)-1, and tissue inhibitors of metalloproteinase (TIMP)-1; (A) semi-qRT-PCR, (B) real-time PCR, (C) western blotting, and (D) enzyme-linked immunosorbent assay (ELISA). The impact of various light emitting diodes (LEDs) at wavelengths (dose: 1 J/cm^2) of 940±2, 850±3, 630±8, 595±2, 580±4, 525±4, 480±7, and 410±10 nm on mRNA and protein levels of TGF-β1, COL-I, COL-III, MMP-1, and TIMP-1; (E) semi-qRT-PCR, (F) real-time PCR, (G) western blotting, and (H) ELISA. *p<0.05, **p<0.01 vs. normal control.

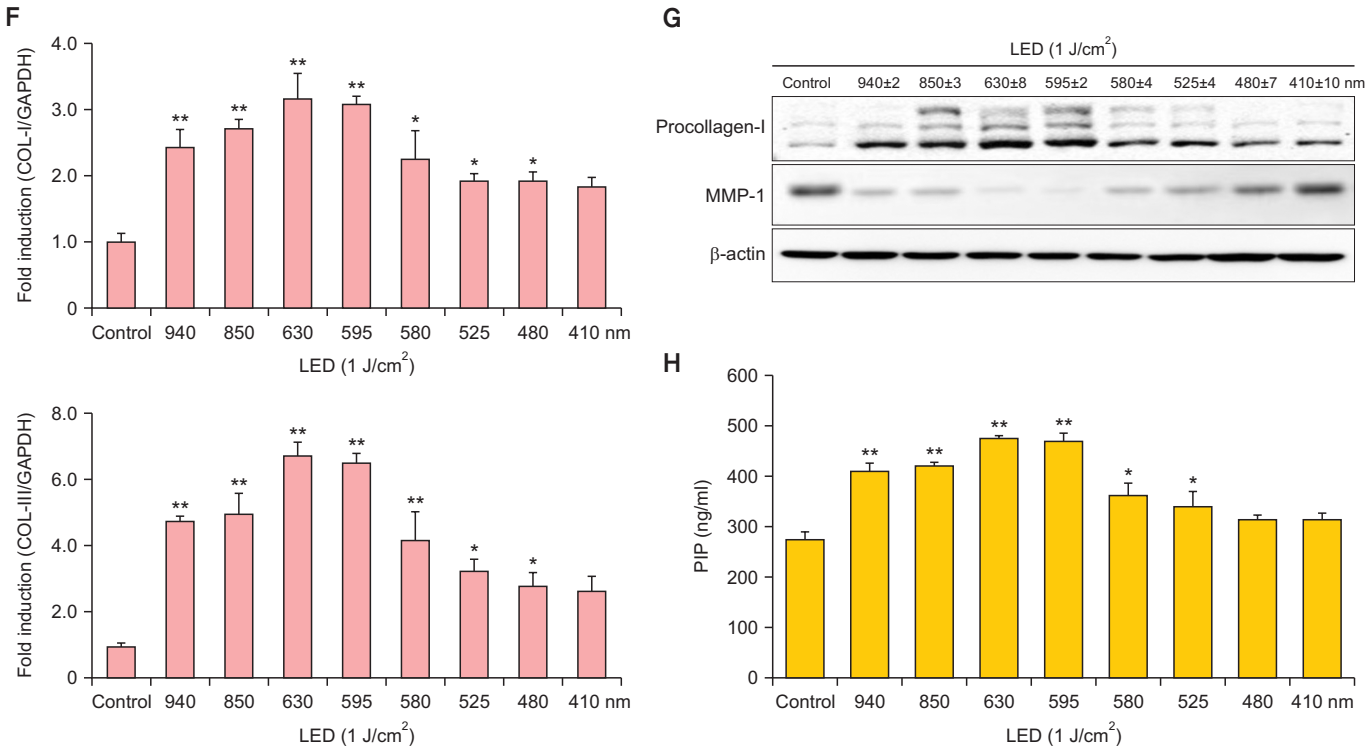

Fig. 2. Continued.

and COL-III increased while those of MMP-1 decreased at the wavelengths 940±2, 850±3, 630±8, 595±2 at a dose of 1 J/cm^2 (Fig. 2E). Among the LED wavelengths (dose, 1 J/cm^2), 940±2, 850±3, 630±8, and 595±2 nm showed significantly increased mRNA levels of both COL-I and COL-III (p<0.01 vs control) (Fig. 2F). On western blot analysis, type I procollagen expression increased while MMP-1 expression decreased with LED irradiation at the wavelengths 940±2, 850±3, 630±8, 595±2, 580±4, 525±4, and 480±7 nm at a dose of 1 J/cm^2 (Fig. 2G). Type I procollagen synthesis after LED irradiation (dose, 1 J/cm^2) at the wavelengths 940±2, 850±3, 630±8, and 595±2 nm also showed a significant difference (p<0.01) (Fig. 2H).

Effect of the MT1/2 receptor antagonist luzindole in combination with melatonin stimulation and LED irradiation on the regulation of collagen and MMPs in HDFs

As MT1 activation was detected in the HDFs, we subsequently tested whether the regulation of collagen and MMPs was mediated by the melatonin receptor. For this purpose, the MT1/2 receptor antagonist luzindole was added to the culture medium at a concentration of 10 μM. Initially, the HDFs were subjected to melatonin stimulation at a dose of 50 nM. Thereafter, the HDFs were subjected to LED irradiation (dose, 1 J/cm^2) at the wavelengths 940±2, 850±3, 630±8, and 595±2 nm, which induced a significant increase in COL-I and COL-III expression.

Luzindole treatment (dose, 10 μM) combined with melatonin (dose, 50 nM) decreased the mRNA levels of COL-I to a greater extent than did melatonin stimulation alone (Fig. 3A, B). Western blot analysis showed that the combined treatment of HDFs with luzindole and melatonin also decreased the expression of type I procollagen to a greater extent than did melatonin stimulation alone (Fig. 3C). Type I procollagen synthesis after luzindole-melatonin co-stimulation and melatonin stimulation alone showed significant differences (p<0.01) (Fig. 3D).

Luzindole treatment (dose, 10 μM) combined with LED irradiation (dose, 1 J/cm^2; wavelengths, 940±2, 850±3, 630±8, and 595±2 nm) decreased the mRNA levels of COL-I to a greater extent than did LED stimulation alone (Fig. 4A, B). Western blot analysis and ELISA also revealed that the combination of luzindole and LED irradiation (dose, 1 J/cm^2; wavelengths, 940±2, 850±3, 630±8, and 595±2 nm) decreased the expression of type I procollagen to a greater extent than did LED stimulation alone (p<0.01) (Fig. 4C, D).

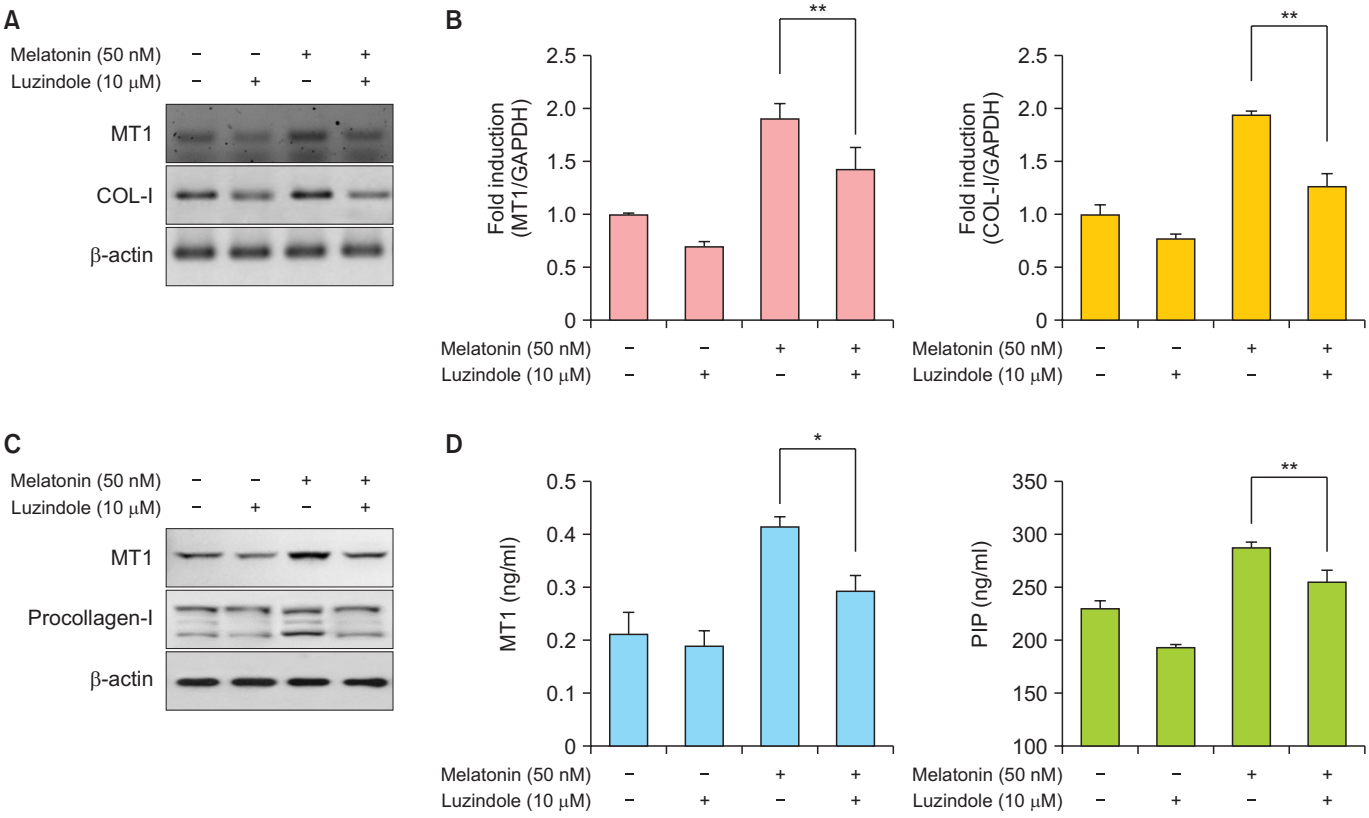

Fig. 3. Regulation of collagen synthesis identified in the human dermal fibroblasts (HDFs) with MT1/2 antagonist luzindole (dose, 10 μm). The impact of melatonin stimulation (dose, 50 nM) with and without luzindole on mRNA and protein levels of melatonin receptor 1 (MT1) and collagen (COL)-I; (A) semi-qRT-PCR, (B) real-time PCR, (C) western blotting, and (D) enzyme-linked immunosorbent assay (ELISA). *$p<0.05$, **$p<0.01$ vs. normal control.

DISCUSSION

The interruption of collagen synthesis and up-regulation of MMP-1, the enzyme related to the turnover of skin collagen, are the main characteristics that differentiate aged skin from younger skin[27]. Numerous LED-based studies have aimed to improve these characteristics and have succeeded in increasing collagen synthesis and reducing MMP-1, not to mention producing clinical improvements, especially with irradiation at the wavelengths 590 and 633 nm in combination with light at 830 nm[20,21,28,29]. In another study, a 940-nm diode laser stimulated the proliferative capacity and cell differentiation of human fibroblasts in relation to wound healing[30]. In the current study, LED irradiation of HDFs at wavelengths 595, 630, 850, and 940 nm produced a significant increase in collagen synthesis and down-regulation of MMP-1, which were consistent with previous study findings.

Several mechanisms have been proposed to explain the action of LED therapy or LLLT. Mitochondria have been considered the primary target of light therapy, which leads to increased adenosine triphosphate (ATP) production via cytochrome C oxidase, the terminal enzyme of the electron transport chain, and alteration of reactive oxygen species (ROS)[31]. Increased ATP subsequently triggers metabolic pathways, and changes in ROS activate numerous intracellular signalling pathways, including nucleic acid and protein synthesis as well as cell cycle progression[32]. In addition, LLLT has been reported to have an effect on the regulation of several genetic transcription factors and gene expressions associated with cell proliferation, inhibition of apoptosis, and energy metabolism and respiratory chain[31]. In our study, LED irradiation at the wavelengths 595, 630, 850, and 940 nm induced collagen synthesis and MMP-1 inhibition, which significantly decreased with the addition of the MT1/2 receptor antagonist luzindole. Melatonin presents effects that are common with LLLT, such as anti-oxidative, anti-inflammatory, and immunomodulatory actions, not to mention cutaneous effects,

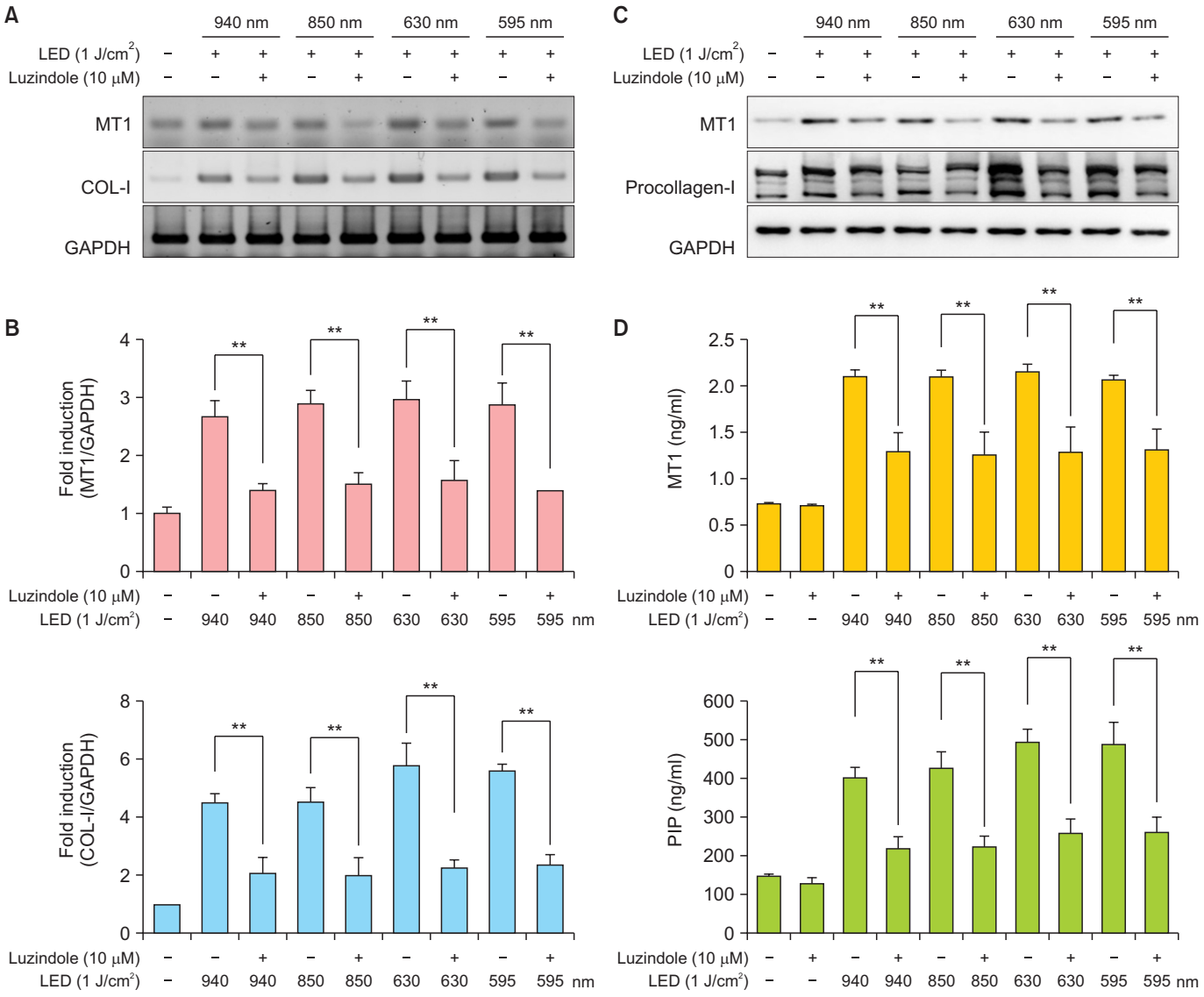

Fig. 4. Regulation of collagen synthesis identified in the human dermal fibroblasts (HDFs) with MT1/2 antagonist luzindole (dose, 10 μm). The impact of various light emitting diodes (LEDs) at wavelengths (dose: 1 J/cm^2) of 940±2, 850±3, 630±8, 595±2, 580±4, 525±4, 480±7, and 410±10 nm with and without luzindole on mRNA and protein levels of melatonin receptor 1 (MT1) and collagen (COL)-I; (A) semi-qRT-PCR, (B) real-time PCR, (C) western blotting, and (D) enzyme-linked immunosorbent assay (ELISA). $^{**}p<0.01$ vs. normal control.

including enhanced collagen synthesis and reduced MMP-1[24]. The TGF-β1/SMAD signalling pathway also plays a role in LLLT-induced rejuvenation[33]. In our study, melatonin stimulation of HDFs increased the mRNA expression of TGF-β1. In addition, the MAPK/ERK pathway, which plays a crucial role in the regulation of fibroblast migration under light stimulation, is also representative of melatonin activity[23,33-35]. Stress-activated protein kinases/c-Jun N-terminal kinase, p38 mitogen-activated protein kinase, and extracellular regulated protein kinases are well-characterized subfamilies of MAPK[35]. Based on our results as well as the common cutaneous effects and relationship between LLLT and melatonin, we propose that the MT1 pathway plays a crucial role in photorejuvenation. Perhaps, LED irradiation may have up-regulated the melatonin hormone itself or an MT1 agonist, or may have directly stimulated the MT1 receptor. However, this is speculative as we did not detect the melatonin hormone or MT1 agonist to confirm or refute this theory.

HDFs, which play a major role in skin rejuvenation, are

located below the epidermis of the skin. Therefore, LED light must first penetrate the epidermis and be less absorbed by the surrounding dermal components to reach the fibroblast. The absorption and scattering of light in tissues are wavelength dependent[23,33]. The major cutaneous chromophores, such as haemoglobin and melanin, show high absorption bands at wavelengths shorter than 600 nm[33]. Water, on the other hand, shows significant absorption at wavelengths longer than 1,150 nm[33]. As a result, wavelengths between 600 and 1,150 nm form a so-called optical window which allows effective light transfer to the dermal fibroblasts[33]. The LED wavelengths 595, 630, 850, and 940 nm, that induced MT1 activation leading to collagen synthesis in our study, are therefore considered suitable treatment options with clinical applications.

Our findings revealed that LED irradiation of HDFs at multiple wavelengths (595, 630, 850, and 940 nm) induced significant melatonin receptor activation. In contrast, irradiation of the eye with a specific bandwidth of blue light from 446 to 477 nm showed significant plasma melatonin suppression[18]. LED with longer wavelengths, including those in the infrared spectrum, revealed no significant suppression[18,36]. Unlike the skin, light stimulation of the retina results in the transmission of signals to the thalamus via the optic nerve and optic tract[37]. The hypothalamus co-ordinates biological clock signals and directs the pineal gland to secrete melatonin. In addition, different types of cells represent different action spectra in response to light[16,19]. While membrane receptor MT2 is primarily found in the retina, human skin expresses a strong bias towards MT1[14,38]. Based on our results, we suggest that human skin may show a melatoninergic response to light distinct from that shown by the eye. Nevertheless, further in vivo evaluation of the effects of LED irradiation in a large population of participants is required to strengthen this observation.

In conclusion, our study revealed that LED irradiation of HDFs at the wavelengths 595, 630, 850, and 940 nm showed significant collagen synthesis and MMP-1 inhibition. These rejuvenative effects were mediated by MT1 activation. This suggests the possibility of the melatoninergic pathway in photorejuvenation. Additionally, the melatoninergic response of the HDFs differed from that of the eye. Multiple LED wavelengths could stimulate MT1 in HDFs, whereas blue light irradiation alone could suppress plasma melatonin secretion in the eye. This suggests that the melatoninergic response of the human skin to light may be distinct from that of the eye.

SUPPLEMENTARY MATERIALS

Supplementary data can be found via http://anndermatol.org/src/sm/ad-21-092-s001.pdf.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Soo Hyeon Bae, https://orcid.org/0000-0002-3124-3326
Jun Hyeong Park, https://orcid.org/0000-0002-5970-4024
Soo-Hyun Kim, https://orcid.org/0000-0002-8711-0557
Sook Jung Yun, https://orcid.org/0000-0003-4229-5831
Jae Gwan Kim, https://orcid.org/0000-0002-1010-7712
Jee-Bum Lee, https://orcid.org/0000-0002-1477-4037

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