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ORIGINAL ARTICLE

TERM

Pigmentation Effect of Electromagnetic Fields at Various Intensities to Melanocytes

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Melanogenesis is the biological process that results in the synthesis of skin pigment of melanin and it has various functions in living systems and is synthesized by the melanosome within the melanocytes. A variety of physical treatments are used to promote melanin production in the melanocytes for pigmentation control. The purpose of this study was to evaluate the intensity-dependent effect of extremely low-frequency electromagnetic fields (ELF-EMFs) on melanogenesis by melanocytes *in vitro*. Melanocytes were exposed to ELF-EMFs at a frequency of 50 Hz and at intensities in the range of 0.5–20 G over 4 days. The results of lactate dehydrogenase assay showed that there were no significant differences between cells exposed to 0.5 G or 2 G groups and the controls. The melanin contents increased 1.2–1.5-fold in cells exposed to ELF-EMFs and tyrosinase activity increased 1.3-fold in cells exposed to ELF-EMFs, relative to the controls. Also, exposure to ELF-EMFs was associated with activation in cyclic-AMP response element binding protein and microphthalmia-associated transcription factor (MITF) was up-regulated. Up-regulation of MITF induces the expression of melanogenesis-related markers, such as tyrosinase, tyrosinase-related protein (TRP)-1, TRP-2. In conclusion, the present study showed that the exposure to ELF-EMFs at low intensities can stimulate melanogenesis in melanocyte, and these results may be used to a therapeutic devices for inducing repigmentation in vitiligo patients.

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Key Words: Extremely low-frequency electromagnetic fields; Melanogenesis; p-AMP response element binding protein; Microphthalmia-associated transcription factor

INTRODUCTION

Melanogenesis is the biological process that results in the synthesis of skin pigment or melanin. Melanin has various functions in living systems and is synthesized by the melanosome within the melanocytes [1]. Many researchers have recently focused on ameliorating diseases caused by hypopigmentation of the skin or hair. Vitiligo is an acquired pigmentation disease that results from the loss of functioning melanocytes [2].

A variety of physical treatments are used to promote melanin production in the melanocytes of vitiligo patients. Narrowband ultraviolet B (NB-UVB) phototherapy is one vitiligo treatment. NB-UVB is also known to stimulate the migration of melanocytes and to induce the secretion of cytokines involved in mitogenesis, melanogenesis, and melanocyte migration. However, this method occasionally causes side effects such as pruritus and erythema [3]. In addition to this method, vitiligo therapies use helium neon and 308 nm excimer lasers [4,5].

It is widely known that extremely low frequency electromagnetic fields interact with biological systems and affect health [6]. Moreover, it has been suggested that extremely low-frequency electromagnetic fields (ELF-EMFs) influence numerous types of cells and processes including cell migration, differentiation, apoptosis, and stress responses [7]. We have previously reported that exposure to ELF-EMFs modulates the osteogenic differentiation of hBM-MSCs by increasing the expression of alkaline phosphatase and osteogenesis-related markers [8] and induces neural differentiation in hBM-MSCs accompanied by higher expression of neurogenesis-related markers [9].

But, some investigator reported that the electromagnetic radiation (900 MHz, SAR 2 mWg^{-1}) did not affect to skin at the point of cellular stress and pigmentation [10]. But electromagnetic radiation and electromagnetic field are different physical area, therefore, the results of each stimulations could not the same. In this study, we will be used the extremely low frequency of electromagnetic fields instead of electromagnetic radiation.

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Melanoblasts, the precursors of melanocytes, are derived from neural crest cells and differentiate into melanocytes after movement to specific sites and proliferation. As mentioned above, ELF-EMFs are known to stimulate neural differentiation and melanocytes are, ultimately, derived from neural crest cells. We therefore hypothesized that ELF-EMFs have the potential to increase melanogenesis and performed this study.

Here, we studied the intensity-dependent effect of ELF-EMFs on melanocytes. Melanocytes were exposed to ELF-EMFs at intensities between 0.5 G and 20 G at a frequency of 50 Hz over 4 days. Melanogenesis was assessed by a variety of analytical means, such as melanin content assay, tyrosinase assay, reverse transcription polymerase chain reaction (RT-qPCR), western blotting, and immunohistochemical staining.

MATERIALS AND METHODS

Cell culture

Melanoblasts were purchased from Creative Bioarray and maintained in Medium 254 (M254, Invitrogen, Waltham, MA, USA) containing PMA-free human melanocyte growth supplement (HMGS-2, Invitrogen, Waltham, MA, USA) at 37°C in an incubator within a 5% humidified atmosphere. The culture medium was changed every 2–3 days, and the cells were passaged 3 to 7 times. To induce differentiation, melanoblasts were exposed to melanocyte differentiation induction medium consisting of M254, HMGS-2, 10 nM alpha-melanocyte stimulating hormone (α-MSH, Sigma Aldrich, St. Louis, MO, USA), 10 nM 12-O-Tetradecanoyl -phorbol-13-acetate (TPA, Sigma Aldrich, St. Louis, MO, USA), and 20 uM Forskolin (Sigma Aldrich, St. Louis, MO, USA). After 3 days, the differentiation induction medium was replaced with M254, before the cells were exposed to ELF-EMFs.

Exposure of melanocytes to ELF-EMFs

A pair of Helmholtz coils were operated with an alternating current, thus generating ELF-EMFs. The ELF-EMF device was placed in an incubator at 37°C and 5% CO_2, and the field was set to different intensities, namely, 0.5, 2, 10, 20 G, at a frequency of 50 Hz and for 30 mis each day over 4 days. Controls were cultured at a separate location to avoid exposure to ELF-EMFs.

Analysis of cytotoxicity

A microscope was used to assess changes in cell morphology under ELF-EMF conditions. Images of cell morphology were taken after 4 days of ELF-EMF exposure.

To evaluate cytotoxicity, a lactate dehydrogenase (LDH) assay was conducted on media collected after cell culture with an LDH-LQ kit (Asan Pharmaceutical Inc., Asan, Korea). The collected media were placed in a 96-well plate, followed by the addition of working solution, mixing, and incubation at room temperature for 30 min. The reaction was terminated with stop solution and then absorbance was measured at 490 nm.

Melanin content assay

The melanin content of cells cultured under ELF-EMFs was determined by a slightly modified method. The cells were seeded in 6-well plates at 1×10^4 cells/well. After exposure to ELF-EMFs, the culture media were removed and the cells were solubilized 10% DMSO dissolved in 1 N NaOH and boiled at 100°C for 30 min. After boiling, the cells were centrifuged at 15000 rpm for 15 min. Melanin content in the supernatant was measured at 405 nm with an ELISA plate reader (Spectrum Analyzer. Victor 1420-050, PerkinElmer Life Science, Turku, Finland).

Tyrosinase activity assay

Tyrosinase activity in cells cultured under ELF-EMFS was determined by a slightly modified method. The cells were seeded in 6-well plates at 1×10^4 cells/well. After exposure to ELF-EMFs, the culture media were removed and the cells were washed with phosphate buffered saline (PBS) and lysed with 10% triton X-100 (Sigma Aldrich, St. Louis, MO, USA). The cells were centrifuged at 15000 rpm for 15 min and the supernatant was used. In order to use the same amount of protein, a BCA assay was performed (Thermo Fisher Scientific, Waltham, MA, USA). After the BCA assay, L-DOPA (Sigma Aldrich, St. Louis, MO, USA) in sodium phosphate buffer (10 mM) was added to the supernatant, which was then incubated for 30 min at 37°C. Absorbance was measured at 475 nm with an ELISA plate reader (Spectrum Analyzer. Victor 1420-050, PerkinElmer Life Science, Turku, Finland).

Quantitative reverse transcription polymerase chain reaction

Total cellular RNA was isolated from the cells using TRIzol (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. The concentration and purity of total RNA were measured by Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). A total of 2 μg of RNA was used for complementary DNA (cDNA) synthesis by reverse transcription with an Advantage RT-PCR kit (Clontech, Palo Alto, CA, USA). The cDNA was subjected to PCR amplification for tyrosinase, tyrosinase-related protein 2 (TRP-2), and microphtalmia-associated transcription factor (MITF), and the primer sequences are listed in Table 1. Band images were obtained with Molecular Imager ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA). For the quantitative analysis of RT-qPCR band images, Image J software

(National Institutes of Health, Bethesda, MD, USA) was used.

Western blotting

Cells were washed with PBS, extracted with sample buffer consisting of 5% 2-mercaptoethanol, 10% glycerol, and 0.1 mg/mL bromophenol blue in Tris-HCl, pH 6.8, and boiled at 100°C for 5 min. To load the same amount of protein, a BCA assay was performed, and the same amount of protein (20–40 μg) was separated by electrophoresis on 8–10% SDS-PAGE and transferred onto a nitrocellulose membrane. Primary antibodies for β-actin (Sigma Aldrich, St. Louis, MO, USA), tyrosinase, TRP-1, MITF (Santa Cruz, Dallas, TX, USA), CREB, p-CREB (Cell Signaling, Danvers, MA,USA) and secondary antibodies such as anti-rabbit, anti-mouse (Cell Signaling, Danvers, MA, USA), and anti-goat (Santa Cruz, Dallas, TX, USA) IgG were used. Band images were obtained with the ECL system (Thermo Fisher Scientific, Waltham, MA, USA) and Molecular Imager ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA). Image J software was used for the quantitative analysis of Western blot band images.

Immunohistochemistry

Cells cultured on a cover slip were treated with 4% paraformaldehyde for 20 min at 4°C and then washed with 10 mM Tris-HCl buffer. The fixed cells were incubated with anti-HMB45 and anti-tyrosinase (Abcam, UK, at 1:1000 dilution) and developed with the EnVision+ kit (DAKO, Copenhagen, Denmark) as a dextran polymer system.

Statistical analysis

Results are reported as the mean±standard error and each experiment was repeated at least three times. Data was analyzed by Student's t-test and one-way analysis of variance. The difference between means was considered to be significant for $p<0.05$ ($^{*}p<0.05$, $^{**}p<0.01$). Graphical representations were produced with the help of Sigmaplot 2001 software.

RESULTS

Figure 1 shows the morphology of melanocytes after exposure to ELF-EMFs over 4 days. Cells showed a bipolar dendrit-

Table 1. Melanogeneic-related primers sequences for RT-PCR

Genes	Upstream primer sequence	Downstream primer sequence
GAPDH	5'-ACC ACA GTC CAT GCC ATC AC-3'	5'-TCC ACC ACC CTG TTG CTG TA-3'
Tyrosinase	5'-CTC AAA GCA GCA TGC ACA AT-3'	5'-GCC CAG ATC TTT GGA TGA AA-3'
TRP-2	5'-TTC GGC AGA ACA TCC ATT CC-3'	5'-TTG GCA ATT TCA TGC TGT TTC-3'

RT-qPCR: quantitative reverse transcription polymerase chain reaction, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, TRP-2: tyrosinase-related protein 2

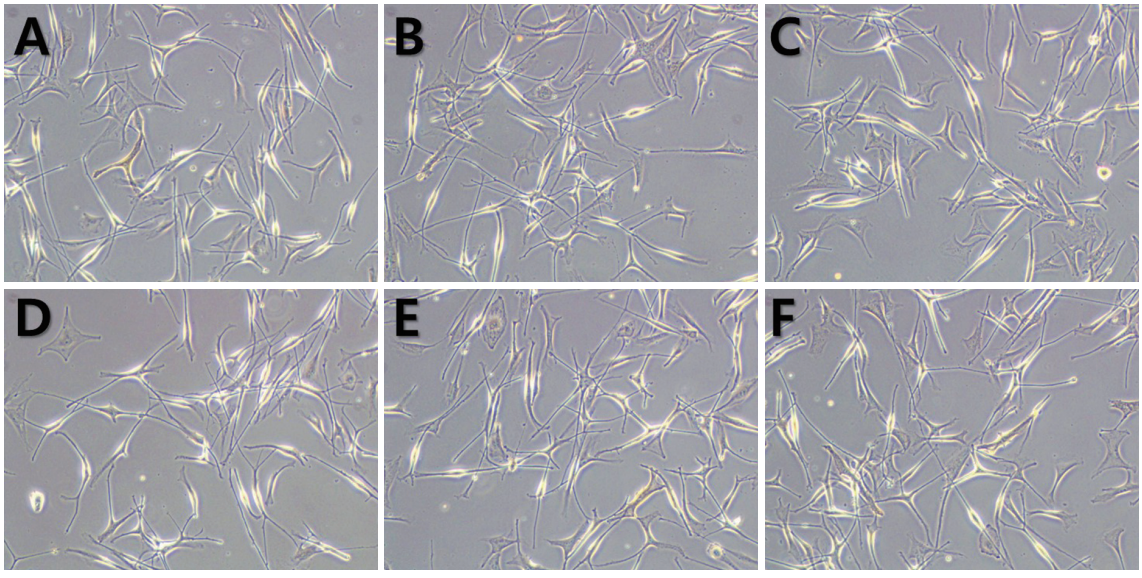

Figure 1. Images of cell morphology of the melanocytes after stimulation with ELF-EMFs over 4 days. All cells were cultured in M254 media. Before exposure to ELF-EMFs, melanoblasts were treated with melanocyte differentiation induction medium for 3 days. After being cultured in the induction medium, the cells were exposed to ELF-EMFs. The α-MSH cells were cultured in M254 medium, to which 5 nM MSH had been added. (A) Control, (B) α-MSH, (C) 0.5 G, (D) 2 G, (E) 10 G, (F) 20 G. Original magnification: ×100. α-MSH: alpha-melanocyte stimulating hormone, ELF-EMFs: extremely low-frequency electromagnetic fields.

ic network and multipolar morphology irrespective of the experimental ELF-EMF conditions. Also, these morphological features were maintained without cell death.

To assess cell damage, the LDH assay was performed. LDH is an oxidative enzyme, is present in the cell membrane and cytoplasm, and is released from cells into culture media after cell damage. The results of this assay showed that there were no significant differences between cells exposed to 0.5 G or 2 G groups and the controls. However, there was a small difference between cells exposed to 10 G or 20 G and the controls. Hence, low intensity ELF-EMFs did appear to be cytotoxic (Fig. 2).

In order to test whether ELF-EMFs induce melanogenesis, the amount of intracellular melanin was determined by a melanin content assay after exposure to ELF-EMFs over 4 days.

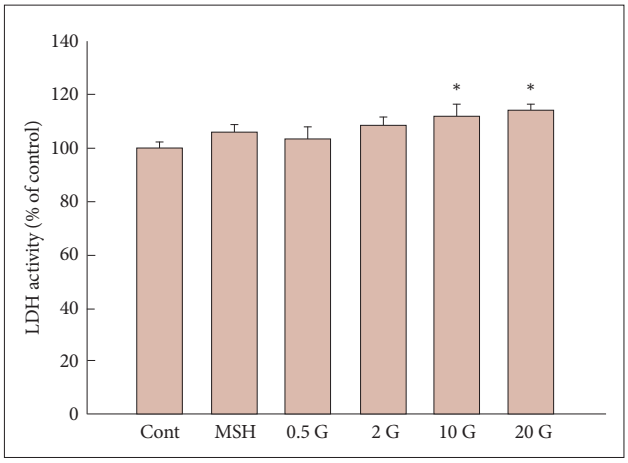

Figure 2. Cytotoxicity of ELF-EMFs exposure over 4 days in melanocytes. Cytotoxicity was measured by LDH assay. Each bar represents the mean±SE of independent experiments performed in triplicate (n=3). *p<0.05 compared with control. LDH: lactate dehydrogenase, MSH: melanocyte stimulating hormone, ELF-EMFs: extremely low-frequency electromagnetic fields.

This revealed that melanin contents increased 1.2–1.5-fold in cells exposed to ELF-EMFs. In particular, cells exposed to 0.5 G and 2 G exhibited a large increase, relative to the controls. Moreover, the melanin content of cells exposed to 0.5 G and 2 G also increased, relative to cells that were exposed to MSH. Therefore, ELF-EMF exposure led to an increase in melanogenesis (Fig. 3A).

To establish that ELF-EMFs induce melanogenesis, tyrosinase activity was determined by tyrosinase activity assay after exposure to ELF-EMFs over 4 days. Crucially, melanogenesis is regulated by tyrosinase activation. This showed that tyrosinase activity increased 1.3-fold in cells exposed to ELF-EMFs, relative to the controls. Therefore, ELF-EMFs induced an increase in melanogenesis in melanocytes (Fig. 3B).

Following studies of cell morphology, melanin content, and tyrosinase activity, the mRNA levels of melanogenesis-related genes (i.e., tyrosinase, TRP-1, MITF) after exposure to ELF-EMFs over 4 days were quantified by RT-qPCR. This revealed that the expression of melanogenesis-related genes increased more than 3-fold in cells exposed to ELF-EMFs relative to the controls. Moreover, there were no significant differences between cells exposed to different intensity ELF-EMFs (Fig. 4).

In addition, immunohistochemical staining was performed with anti-HMB45, a melanosome-specific antigen, anti-tyrosinase after exposure to ELF-EMFs over 4 days. Both melanogenesis-related markers were expressed in cells exposed to ELF-EMFs in comparison with the controls (Fig. 5).

Based on the results of RT-qPCR and immunohistochemical staining, the expression of melanogenesis-related proteins was measured by Western blotting after exposure to ELF-EMFs over 4 days. As shown in Figure 6, relative to the controls, the expression of all melanogenesis-related proteins increased in cells that were exposed to ELF-EMFs. In particular, tyrosinase

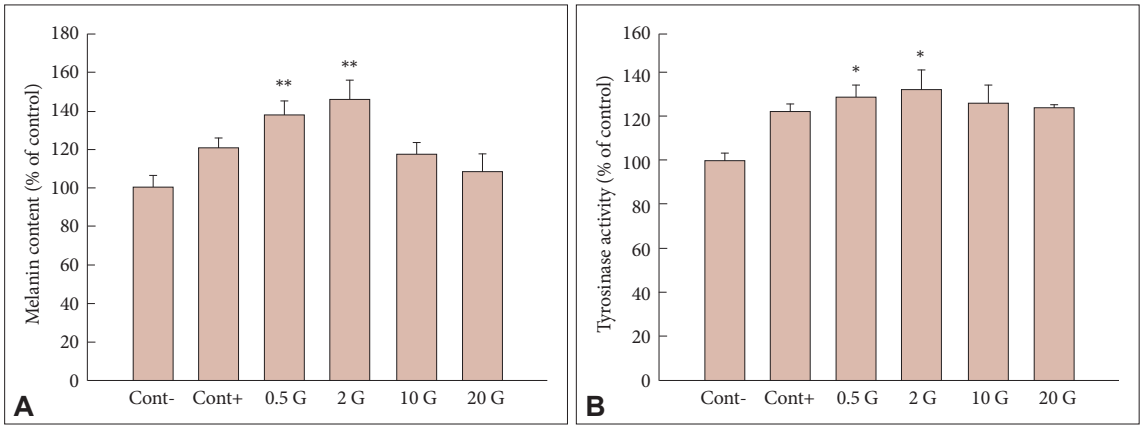

Figure 3. Effect of ELF-EMFs exposure over 4 days on melanogenesis in melanocytes. Melanogenesis was assessed by melanin content assay and tyrosinase activity assay. (A) The melanin content assay determined the cellular melanin concentration. (B) The tyrosinase activity assay measured cellular tyrosinase activity. Each bar represents the mean±SE of independent experiments performed in triplicate (n=3). *p<0.05, **p<0.01 compared with control. ELF-EMFs: extremely low-frequency electromagnetic fields.

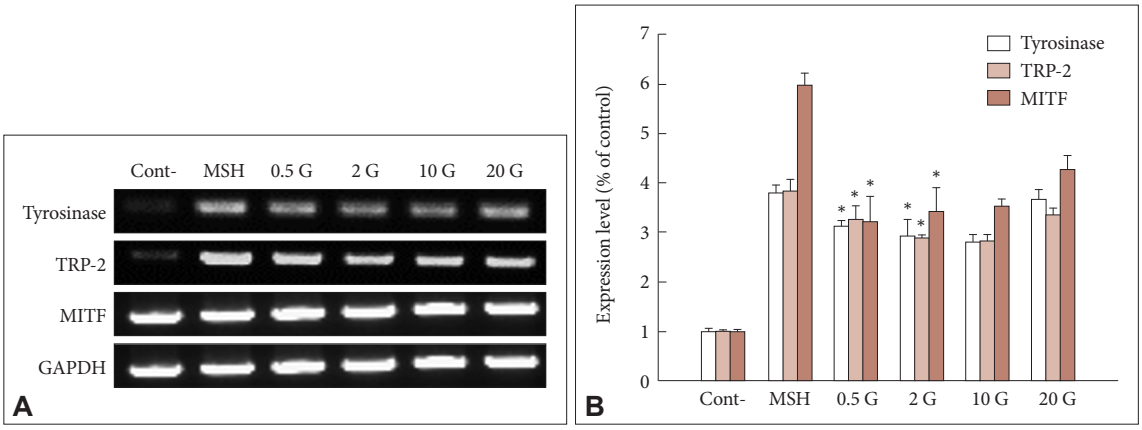

Figure 4. Gene expression levels in melanocytes determined by RT-PCR after exposure to EMFs for 4 days. Melanogenesis-related proteins (tyrosinase, TRP-2, MITF) were studied. (A) Band images of melanogenesis-related genes following RT-qPCR. (B) Quantitative analysis of mRNA expression levels of melanogenesis-related genes after ELF-EMFs exposure, relative to GAPDH. Each bar represent the mean±SE of independent experiments performed in triplicate (n=3). *p<0.05 compared with control. RT-qPCR: reverse transcription polymerase chain reaction, EMFs: electromagnetic fields, TRP-2: tyrosinase-related protein 2, MITF: microphthalmia-associated transcription factor, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, MSH: melanocyte stimulating hormone.

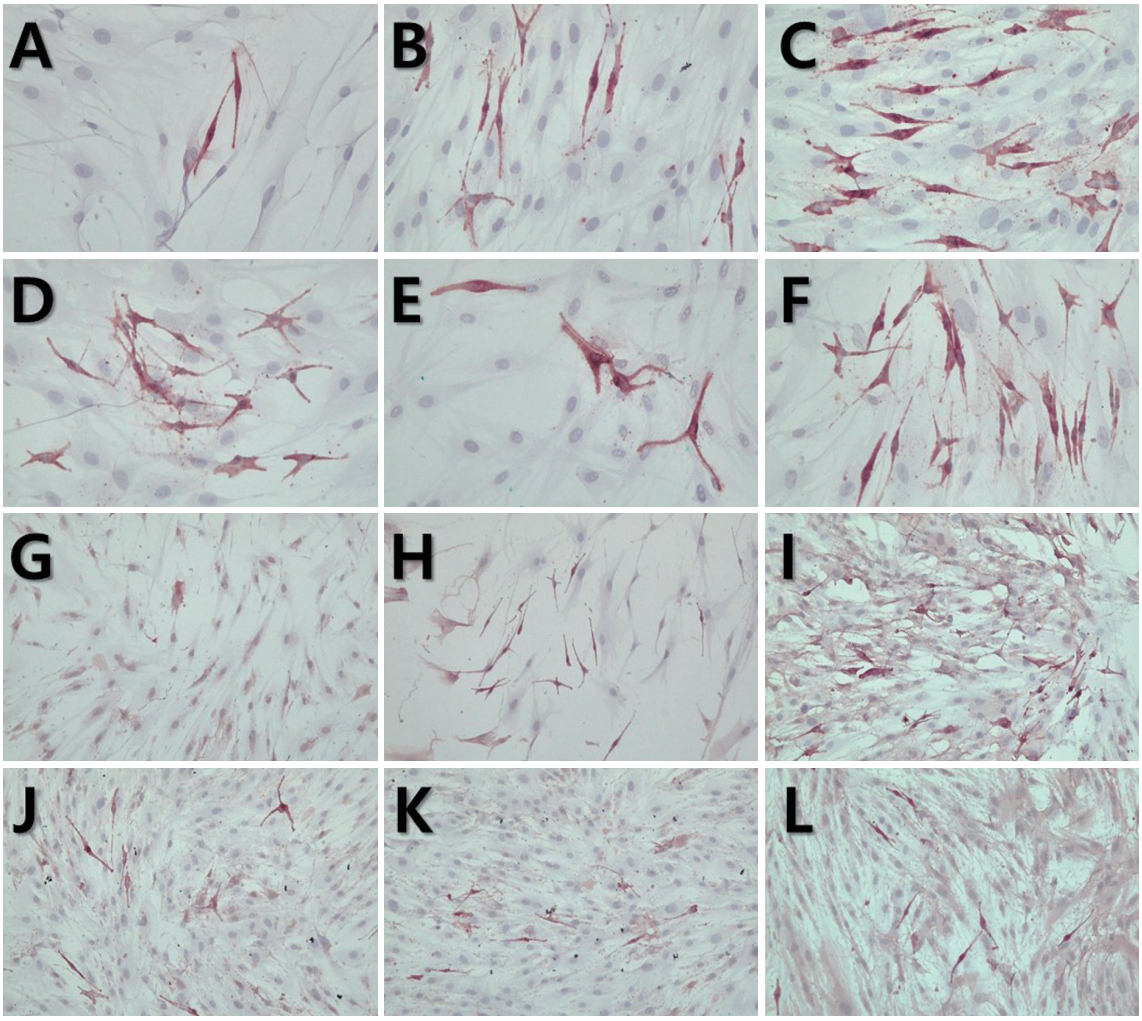

Figure 5. Phase-contrast photograph of human skin melanocytes attached to a glass surface and subjected to immunohistochemical staining of HMB45 and tyrosinase after exposure to EMFs over 4 days. (A-F) HMB45, (A) control, (B) α-MSH, (C) 0.5 G, (D) 2 G, (E) 10 G, (F) 20 G. (G-L) Tyrosinase: (G) control, (H) α-MSH, (I) 0.5 G, (J) 2 G, (K) 10 G, (L) 20 G.

expression increased more than 2-fold, TRP-1 expression increased more than 1.3-fold, and MITF expression increased more than 5-fold. In cells exposed to 0.5 G and 2 G, tyrosinase and TRP-1 expression levels increased especially, that is, by more than 4-fold relative to the controls (Fig. 6).

We also assessed the activation of CREB signaling. CREB is a transcription factor that regulates MITF expression. As shown in Figure 7, p-CREB levels increased more than 2-fold in cells exposed to ELF-EMFs. Cells exposed to 0.5 G and 2 G displayed especially strong expression of this protein, in contrast to cells exposed to 20 G.

DISCUSSION

A variety of approaches have been proposed including broad-band UVB, NB UVB, psoralen plus UVA (PUVA), excimer (351 nm), argon (488514 nm), ruby (694 nm), Nd:YAG (1060 nm), and CO_2 (10600 nm) lasers for inducing of pigmentation [11-14]. Lan et al. [12] reported that the HeNe laser (632.8 nm, 1 J/cm^2, 10 mW) stimulates melanocyte proliferation through enhanced α2β1 integrin expression and induces melanocyte growth through upregulation of the expression of phosphorylated cyclic-AMP response element binding protein (CREB). And, Goldberg et al. [14] used excimer laser and UVB light sources for inducing of melanogenesis. But the side effects of these treatment are mild, transient, localized, erythema, blisters, and perilesional pigmentation has been reported [15].

EMFs have been reported to have biological effects by impinging on cell viability, cell proliferation, cell differentiation, and cell death [16-19]. Moreover, EMFs have the advantage of

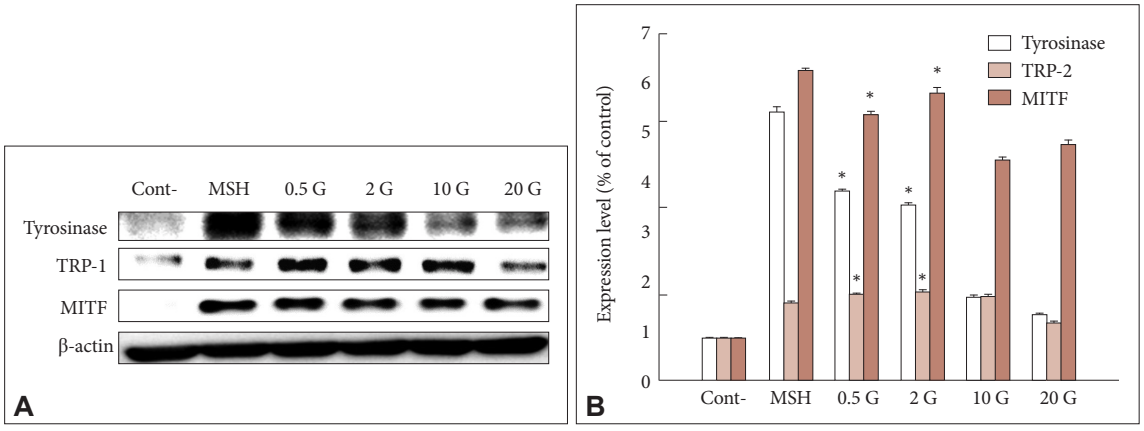

Figure 6. Protein expression levels determined by Western blotting after exposure of melanocytes to EMFs over 4 days. Melanogenesis-related proteins (tyrosinase, TRP-1, MITF) were detected by Western blotting. (A) Band images of melanogenesis-related proteins by Western blotting. (B) Quantitative analysis of the expression levels of melanogenesis-related proteins after exposure to ELF-EMFs. β-actin served as an internal control. Each bar represents the mean±SE of independent experiments performed in triplicate (n=3). *p<0.05 compared with control. ELF-EMFs: extremely low-frequency electromagnetic fields, TRP-1: tyrosinase-related protein 1, MITF: microphthalmia-associated transcription factor.

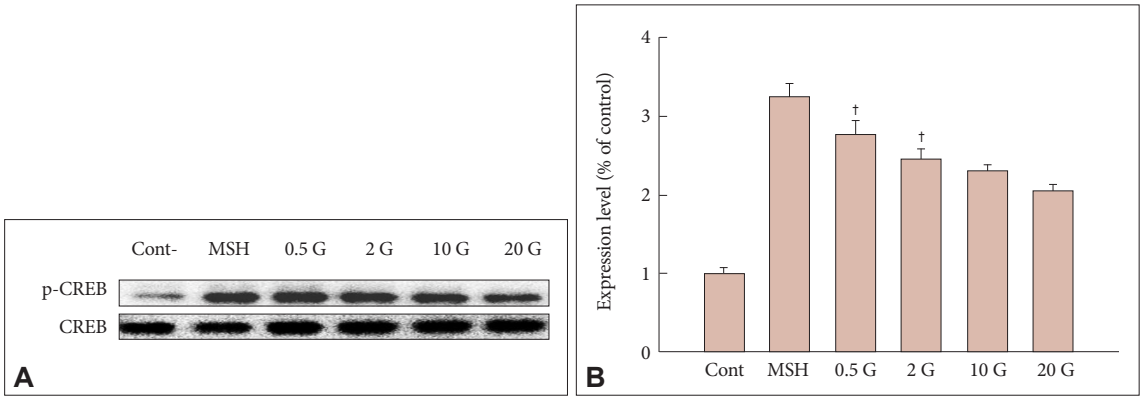

Figure 7. p-CREB expression level determined by Western blotting after exposure of melanocytes to EMFs over 4 days. CREB activation was detected by Western blotting. (A) Band images of CREB and p-CREB by Western blotting. (B) Quantitative analysis of the expression levels of CREB and p-CREB after exposure to ELF-EMFs. Each bar represents the mean±SE of independent experiments performed in triplicate (n=3). †p<0.01 compared with control. CREB: AMP response element binding protein, ELF-EMFs: extremely low-frequency electromagnetic fields.

being non-toxic and non-invasive, and, as a consequence, a particularly high number of studies has been performed in field of neurogenesis.

We have previously reported that ELF-EMFs promote neural differentiation of mesenchymal stem cells. In particular, we used RT-qPCR and Western blotting to show that ELF-EMFs stimulate the expression of neural genes and proteins. Thus, our results suggested that ELF-EMFs stimulation significantly promoted neural differentiation of hBM-MSCs [9,20].

Melanoblasts are the precursors of melanocytes and differentiate into melanocytes after migration and proliferation at specific sites such as the dermis, epidermis, and hair follicles. Melanoblasts are derived from neural crest cells, which also give rise to peripheral neurons, glial cells, and, ultimately, melanocytes in precise locations during embryogenesis [21-23].

Dong et al. [11] reported that NB-UVB can be induced melanocyte differentiation from hair follicle-derived neural crest stem cells *in vitro* under various energy (0.2–0.6 J). At this study, NB-UVB increased the expression of tyrosinase and pigmentation related gene during melanocytic differentiation from stem cell as NB-UVB energy increased. However, the migration of stem cells was upregulated at 0.4 J NB-UVB radiation. We therefore speculated that ELF-EMFs may stimulate melanogenesis at the specific intensity.

The goal of this study was to determine the ELF-EMF intensity dependence of melanogenesis and to identify optimal conditions for ELF-EMF-induced melanogenesis in melanocytes. Further, the intensity dependent cytotoxicity of ELF-EMFs was evaluated by cell morphology and LDH assay. This showed that ELF-EMFs are not cytotoxic in melanocytes.

Melanin is synthesized in melanosomes that contain specific enzymes such as tyrosinase, TRP-1, and TRP-2 that are required for melanin production. Tyrosinase is a rate-limiting enzyme in melanogenesis and initially catalyzes the conversion of tyrosine into dopaquinone. TRP-1 and TRP-2 act downstream in the biosynthetic pathway of melanin [24-26]. In melanocytes, increased tyrosinase activity could be achieved by upregulation of tyrosinase gene expression, leading to an increase in the level of the protein.

Intracellular tyrosinase activity increased when melanocytes were exposed to ELF-EMFs at 0.5–20 G, as shown in Figure 3. RT-qPCR and immunohistochemical staining showed that tyrosinase expression increased at 0.5–20 G at the gene level and at the protein level. Moreover, TRP-2 expression as quantified by RT-qPCR increased at 0.5–20 G. Tyrosinase and TRP-1 expression levels increased especially in cells exposed to 0.5 G and 2 G, and to a lesser extent in cells exposed to 10 G and 20 G.

As a melanocyte-specific transcription factor involved in growth, differentiation, function, and survival, MITF is also involved in melanogenesis. Stimulation of cAMP signaling leads to an increase in MITF protein levels and tyrosinase, TRP-1, TRP-2 activation [27-29]. We studied MITF expression by Western blotting and found that MITF expression increases in cells exposed to ELF-EMFs.

Jiang et al. [30] have showed that sesamin induces melanogenesis by upregulating the expression of MITF, tyrosinase, and p-CREB in human melanocytes. Wei et al. [31] have reported that cilostazol enhances the expression of MITF and downstream factors, TRP-1 and tyrosinase, thus inducing increases in tyrosinase activity and melanin content; cilostazol also activated CREB in B16-F10 melanoma cells. According to these studies, the expression and function of MITF is regulated by PKA signaling. Activation of PKA phosphorylates CREB, resulting in the expression of MITF. These multiple signaling cascades have been identified as key factors in the regulation of MITF. In this study, ELF-EMFs led to phosphorylation of CREB, and activated CREB increased MITF expression according to the results of Western blotting. The results of the present study demonstrate that exposure to ELF-EMFs can stimulate melanogenesis in melanocytes.

Conclusion

We have demonstrated that ELF-EMFs can stimulate melanogenesis in melanocytes. Exposure to EMFs led to increases in p-CREB, which resulted in enhanced expression of MITF and downstream factors TRP-1, TRP-2, and tyrosinase. EMFs interact with biological systems without causing cellular damage. Based on these results, we suggest that ELF-EMF devices may be useful in the treatment of hypopigmentation-related diseases. However, the mechanism underlying melanogenesis is still unknown and needs to be the subject of future studies.

Acknowledgements

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There are no animal experiments carried out for this article.

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