Preconditioning with repetitive electromagnetic stimulation enhances activity of bone marrow mesenchymal stem cells from elderly patients through Erk1/2 via nitric oxide

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Abstract. Use of bone marrow aspirate (BMA) is a clinically advantageous cell therapeutic that bypasses the need for elaborate *ex vivo* cell culturing. However, a low level of bone marrow-mesenchymal stem cells (BM-MSCs) in the BMA and weak survival rate of these cells post-transplantation entails an insufficient efficacy *in vivo*. Moreover, stem cell activity in BMA is impaired by age or background diseases. Thus, in order to enrich the BM-MSC pool and improve cell survival, novel cell preconditioning technologies are required. In this study, it has been revealed that the pretreatment of repetitive electromagnetic stimulation (rEMS) is capable of enhancing fibroblastic colony-forming units and cell proliferation in the BM-MSCs, possibly via transient nitric oxide production and extracellular signal regulated kinase 1/2 activation. Notably, this effect was more apparent in stem cells isolated from older patients than from young patients. Furthermore, the rEMS-pretreated cells showed ~53% higher cell survival, compared with the untreated cells, after cell transplantation in mice with no signs of tumorigenesis. Collectively, transient rEMS preconditioning could be utilized to enhance the activity of stem cells and thus, application of rEMS preconditioning to stem cells isolated from older patients is expected to improve the therapeutic effect of stem cells.

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Introduction

Bone marrow-derived autologous stem cell therapy has been proposed as a promising treatment for a variety of incurable degenerative diseases such as graft versus host disease (1), spinal cord injury (2), myocardial infarction (3), autoimmune disease and osteoarthritis (4,5). Currently, stem cell therapy is mainly conducted in one of two ways. One approach is the use of minimally manipulated cells from bone marrow aspirates (BMAs), in which mononuclear cells (MNCs) are isolated by Ficoll-gradient centrifugation and infused without *in vitro* expansion. BMAs are used immediately after cell isolation from patients and bypass the very expensive and elaborate cell culture methods. The other approach is local or systemic transplant of *ex vivo* long-term cultured stem cells, which have the homogenous cell types including mesenchymal stem cells or endothelial progenitor cells and show efficacy in specific diseases.

To date, numerous clinical trials using stem cells have been attempted and the therapeutic effect of stem cell therapy has been demonstrated (6,7). However, when considering that older patients are primarily subjected to stem cell therapy, an insufficient number of stem cells in these patients and the poor viability of transplanted stem cells *in vivo* have been major obstacles in the progression of stem cell therapy to successful therapeutic outcomes (8,9).

In order to enhance stem cell activity and survival in the clinical setting, the preconditioning strategies of minimally manipulated cells from BMA or *ex vivo* cultured stem cells from older patients should be accomplished. Currently, the preconditioning of stem cells has been attempted through gene modification, physical training such as cell straining, or treatment with biological/chemical factors, which is expected to contribute to improving the efficacy of stem cells *in vivo*.

The transcranial application of repetitive electromagnetic stimulation (rEMS) is a non-invasive technique that repeatedly introduces electromagnetic currents in order to stimulate a small area of the brain. Previously, rEMS has been utilized in experimental neurophysiology to explore its therapeutic implications (10) and has been clinically applied

to treat several psychiatric and neurological diseases such as depression, stroke, Parkinson's disease, tinnitus, epilepsy, and pain (11-16). Despite the wide clinical use of rEMS, its effect on stem cells has not yet been fully investigated, with only a few studies reporting the release of second messenger production or cell signaling induced by rEMS (17-19). rEMS was found to increase the affinity of brain-derived neurotrophic factors for tyrosine receptor kinase B (TrkB), which promoted recruitment of phospholipase Cγ1 and Shc/N-Shc to phosphorylated TrkB. This activated downstream extracellular signal-regulated kinase 2 (Erk2) and phosphoinositide 3-kinase in the prefrontal cortex and in lymphocytes (17). An extremely low frequency electromagnetic stimulus was able to activate phosphorylated (p)-ERK and p-SAPK/JNK pathways in human melanocytes (19). rEMS have been shown to activate the cyclic adenosine monophosphate (cAMP)/cAMP response element-binding protein (CREB) pathway in human-derived SH-SY5Y neuroblastoma cells by generating cAMP and the subsequent phosphorylation of CREB (18). Diniz *et al* (20) revealed that electromagnetic field stimulates osteoblast proliferation and differentiation through nitric oxide. These studies described the molecular action of electromagnetic stimulation, suggesting the potential of electromagnetic preconditioning as a treatment.

The approach of the present study is based on the hypothesis that transient rEMS preconditioning of adult stem cells before cell transplantation can enhance their activity, which may be a versatile tool for stem cell therapeutics. In this study, the effects of rEMS treatment on bone marrow mesenchymal stem cells (BM-MSCs) derived from BMA was explored by examining colony-forming efficiency, Erk activation, nitric oxide (NO) production, proliferation and survival. Additionally, the effect of rEMS on cells derived from patients of different ages was comparatively analyzed.

Materials and methods

Cell preparation and culture. Donors comprised young (mean age of 40±10.09 years, n=21) and old (mean age of 69.6±7.4 years, n=30) patients. Patient characteristics are shown in Table SI. Human BMAs (10 ml) were obtained with the written consent of patients and the protocol was approved by the St. Peter's Hospital Institutional Review Board (KPH IRB 2009-001).

Briefly, the aspirates (10 ml) were harvested from the posterior iliac crest of patients with a 20-gauge spinal needle. The samples were centrifuged twice at 363 x g at 4˚C for 10 min to separate MNCs at the interface. Total MNCs were harvested as minimally manipulated BMA cells. For BM-MSC culture, $2x10^6$ of MNCs were cultured in MSC growth medium (MSCGM; Lonza Group, Ltd.) at 37˚C in a humidified atmosphere of 5% CO_2. The growth medium was replaced every other day. The cells were cultured until passage 4 for 1 month. The cells between passages 0 and 3 were used in the experiments. BM-MSCs were characterized by cell surface marker expression and multi-differentiation assays (Table SI; Figs. S1 and S2).

rEMS treatment. Harvested MNCs or primary cultured BM-MSCs ($5x10^6$ cell) were placed at 1 cm from the magnetic coil of an electromagnetic stimulator (TAMAS; CR Technology, Co. Ltd.). The rEMS parameters were as follows: Stimulating frequency of 25 Hz, stimulating pulse intensity at 70% of the maximum power (3 Tesla), inter-train interval of 5 sec and a stimulation time of 5, 10, or 15 min. The untreated control cells were treated in the same manner for the same time period but without rEMS. All stimulations were performed on single-cell suspensions. Immediately after stimulation, the cells were analyzed for Erk activation or plated at a density of $2x10^4$ cells per ml for cell proliferation assays.

Fibroblastic colony-forming unit (CFU-f) assay. MNCs ($2x10^6$; 36,300 cells/cm^2 area, P0) or BM-MSCs ($2x10^3$, 36.3 cells/cm^2 area) were stimulated with rEMS and then incubated in 100 mm at 37˚C in 5% CO_2. After 24 h, the medium was changed to remove non-adherent cells. For the CFU-f assays, the cells were cultured for 10 days and then fixed with 3.7% formaldehyde (Sigma-Aldrich; Merck KGaA) for 20 min at room temperature. The fixed colonies were stained with crystal violet (Sigma-Aldrich; Merck KGaA) for 20 min at room temperature. Colonies were observed by light microscopy (magnification, x40; Olympus Corporation) and a fibroblastic cluster consisting of >50 cells was counted as a colony.

Bromo-2'-deoxyuridine (BrdU) incorporation assay. At 20 h after plating, the cells were incubated with 10 μM BrdU (Sigma-Aldrich; Merck KGaA) for 4 h and fixed with methanol (Merck KGaA) for 10 min at room temperature. Next, the cells were incubated with anti-BrdU antibody (1:50; cat. no. 11170376001, Roche Molecular Diagnostics) for 1 h at room temperature, then with Alexa 488-conjugated goat anti-mouse antibody (1:100; cat. no. 115-545-205 Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature and counterstained with propidium iodide (PI; Sigma-Aldrich; Merck KGaA) for 5 min at room temperature. The cells were mounted with Vectashield mounting medium (Vector Laboratories, Inc.) and observed using an Eclipse Ti fluorescence microscope (Nikon Corporation).

Cell viability assay. Cells were plated at a density of $2x10^5$ cells per ml ($2x10^4$/well) immediately after rEMS treatment. After 24 h, each well was treated with water-soluble tetrazolium salt-1 (WST-1; Takara Bio, Inc.) and incubated for 30 min at 37˚C in 5% CO_2. The optical density was measured at 490 nm using a microplate spectrophotometer (Promega Corporation). Viability was calculated as a relative to the control.

Western blot analysis. BM-MSCs were pretreated with 50 μM PD98059 [mitogen-activated protein kinase/Erk kinase (MEK), inhibitor, Cell Signaling Technology, Inc.], 300 μM (4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (NO scavenger, carboxy-PTIO), or 1 mM L-N^G-monomethyl arginine monoacetate (L-NMMA; NOS inhibitor; Sigma-Aldrich; Merck KGaA) for 1 h before stimulation with rEMS. Immediately after rEMS, the cells were prepared with cell lysis buffer (cat. no. 9803; Cell Signaling Technology, Inc.) and the protein quantification was performed using BCA reagents. Proteins (10 μg) were resolved with 10%

SDS-PAGE and transferred onto a nitrocellulose membrane. To avoid non-specific binding, the membrane was incubated with 1% skim milk for 40 min at room temperature. The membrane was incubated with antibodies specific to p-Erk (1:2,000; cat. no. 9106; Cell Signaling Technology, Inc.) or pan-Erk (1:1,000; cat. no. 9102; Cell Signaling Technology, Inc.) for 16 h at 4˚C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000; cat. no. 0300-0108P; Bio-Rad Laboratories, Inc.) for 1 h at room temperature. The membrane was developed using the Immobilon Western Chemiluminescent HRP Substrate (cat. no. WBKLS0100; EMD Millipore) and visualized by exposure to X-ray film (AGFA, Mortsel).

Measurement of NO production. BM-MSCs at $5x10^4$ cells per ml were plated immediately after 5, 10, or 15 min rEMS treatment and then incubated at 37˚C in 5% CO_2 for 24 h in order to measure NO. Griess reagent kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to quantify nitrite concentration in the culture supernatant. Absorbance was determined using a microplate spectrophotometer at a wavelength of 550 nm.

In vivo transplantation. Six-week-old balb/c nude mice (22-23 g, male, n=6) were purchased from Daehan Bio Link, Co., Ltd. Animals were maintained under a 12-h light/12-h dark illumination cycle in an animal holding room and allowed to acclimatize to the new environment for a period of 1 week before initiation of the experiments. All animals received a standard chow diet and water *ad libitum*. This study was approved by the Ethical Committee for Experimental Animals of Kyung Hee University (approval no. KHMC-IACUC-14-010). All mice were anesthetized intraoperatively using intraperitoneal injections of ketamine (100 mg/kg; Yuhan) and Rumpun (1.2 mg/kg; Bayer).

BM-MSCs ($2x10^6$ cells per mouse) with or without rEMS treatment were labeled with PKH26 red fluorescent cell linker (Sigma-Aldrich; Merck KGaA), mixed with Matrigel (BD Bioscience; Becton, Dickinson and Company), and implanted into the subcutaneous tissue in the dorsal region of mice (Daehan Bio Link, Co., Ltd.). A total of 8 weeks after transplantation, Mouse was sacrificed by CO_2 inhalation

Samples (transplanted cells and Matrigel) were harvested, fixed in 3.7% formaldehyde for 24 h at room temperature and embedded in paraffin or optical cutting temperature compound (Sakura Finetek UK, Ltd.). Paraffin sectioned samples (5 μm) were analyzed after hematoxylin & eosin (Sigma-Aldrich; Merck KGaA) staining (5 min, room temperature). To track the transplanted cells labeled with PKH26, cryosectioned samples were mounted with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc.) and observed using an Eclipse Ti fluorescence microscope.

Statistical analysis. Experiments were repeated three times and data are presented as the mean ± standard error of the mean. Comparisons among three groups were performed using one way analysis of variance followed by Tukey's post hoc test. All data were analyzed using GraphPad Prism software (7.0; GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Age impairs cellular activity and proliferation of stem cells in the BM. In order to examine the effect of age on stem cell activity, BMAs were prepared from young or old patients and then cellular activity was assessed by WST-1 assay ($P<0.01$; Fig. 1A). The activity of total BM-MNCs was decreased in the old patients compared with young patients. When BM-MNCs from the old patients were cultured in MSCGM, CFU-f was found to be decreased and proliferation doubling time was increased, compared with BM-MSCs from the young patients ($P<0.01$; Fig. 1B: Young: 102.71±6.63 days, old: 55.29±11.15 days, Young vs. Old, $P<0.01$; Fig. 1C: Young: 1.88±0.37 days, old: 3.77±0.67 days, Young vs. Old).

This data suggests that as people get older, the stem cell pool and its activity in the bone marrow may be weakened. Also, due to increased population doubling time, the total cumulative cell number to be collected after *ex vivo* culture is less in old compared with young patients. Above all, when taking into consideration that cell transplantation is chiefly required for older patients, the establishment of modulations that enhance stem cell activity in older patients are more important.

Effect of rEMS preconditioning on stem cells is more prominent in aged BMAs. To explore the effect of rEMS on bone marrow cells, MNCs derived from either the BMA of young or old patients were subjected to rEMS treatment for 5 min and their CFU-f values were analyzed and compared (Fig. 2). In the absence of rEMS treatment, the CFU-f of BMAs from young patients was increased compared with BMAs from the old patients. rEMS treatment increased the CFU-f of the BM-MSC pools in both groups. The effect was greater in the BMAs from the old patients, with an ~53.8% increase, compared with a 21.2% increase seen in BMAs from the young patients [The young, -rEMS group: $87.3+12.9/2x10^6$ MNCs, +rEMS group:$105.8+14.6/2x10^6$ MNCs, $P<0.01$ vs. -rEMS group; The Old, rEMS treatment (-): $56.5+11.5/2x10^6$ MNCs, rEMS treatment (+): $86.9+21.52x10^6$ MNCs, $P<0.05$ vs. rEMS treatment (-)].

This finding indicates that rEMS preconditioning is more efficient in BM stem cells from old patients (-rEMS group vs. +rEMS group).

Transient application of rEMS enhances the CFU-f of the BM-MSCs. To explore whether rEMS application affects the cell proliferation and self-renewal capacity of cultured BM-MSCs from the old, rEMS was applied to BM-MSCs (Fig. 3A). The effect of rEMS on BM-MSCs was evaluated using CFU-f (Fig. 3B and C). Transient application of rEMS increased the CFU-f by 27.8±4.8% compared with the untreated control ($P<0.05$; -rEMS group vs. +rEMS group). Additionally, rEMS treatment did not influence specific marker expression of MSCs from the old patients (Fig. S3). In other words, these data support that rEMS preconditioning can increase the self-renewal capacity of stem cells cultured *ex vivo*. Thus, rEMS treatment before stem cell transplantation is expected to enhance the therapeutic gain of BM-MSCs without affecting the characteristics of stem cells.

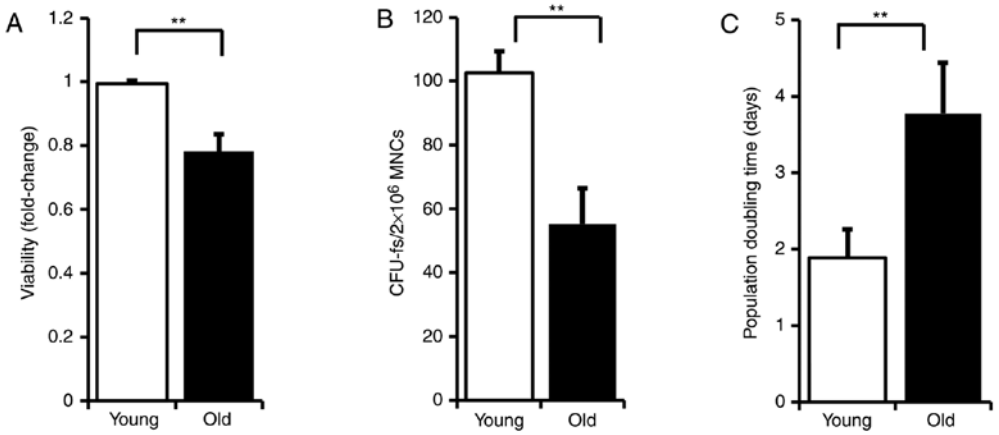

Figure 1. Bone marrow stem cell activity is affected by age. Bone marrow aspirates were collected from young (n=21) and old (n=30) patients, and MNCs were isolated and cultured *in vitro*. (A) Viability (fold change) of BM-MNC (passage 0) was determined by water-soluble tetrazolium salt-1 assay. The activity of MSCs from young patients was set to '1' and the activity of MSCs from old patients was expressed relative to the activity of young patient MSCs (reference to the 'young' group). P<0.01 vs. the MSC from young patients. (B) CFU-F P<0.01 vs. the MSC from young patients and (C) population doubling time was comparatively analyzed. P<0.01 vs. the MSC from young patients. Data represents the mean ± standard deviation; one-way analysis of variance with Tukey's post hoc test. **P<0.01 vs. the Young. Young, MNC from the young patients; Old, MNC from the old patients; BM-MNCs, bone marrow-mononuclear cells; CFU-f, colony forming units; MSC, mesenchymal stem cell.

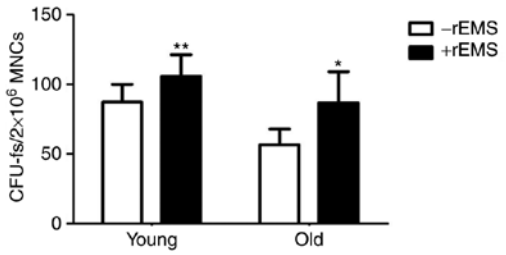

Figure 2. Effect of rEMS treatment on the colony-forming efficiency of MNCs from young and old patients. To evaluate the rEMS-mediated effect on the CFU-f, MNCs derived from the BMAs of the two groups (mean age of young and old patients was 44±10.09 and 68.6±5.97 y, respectively) were stimulated with rEMS for 5 min, and cultured *in vitro*. Their CFU-f efficiencies were compared. The young: P<0.01 vs. -rEMS group. The old: P<0.05 vs. -rEMS group (n=21 in young, n=30 in old). Data represents the mean ± standard deviation; one-way analysis of variance with Tukey's post hoc test. *P<0.05 and **P<0.01 vs. respective -rEMS group. Young, MNC from the young patients; Old, MNC from the old patients; BMA, bone marrow aspirate; rEMS, repetitive electromagnetic stimulation; MNCs, mononuclear cells; CFU-f, colony forming units.

rEMS increases cell proliferation of BM-MSCs, possibly through transient NO production and subsequent Erk1/2 activation. The increase in CFU-f due to transient rEMS treatment led the present study to explore the effect of rEMS on BM-MSC proliferation. rEMS was used to treat BM-MSCs from the old patients at different time lengths. rEMS treatment for 5 or 10 min increased both cell viability (Fig. 4A) and the number of BrdU-incorporating cells in BM-MSCs (Fig. 4B), compared with the untreated controls. However, the effects of rEMS were not observed after 15 min treatment. Moreover, rEMS treatment for 5 min resulted in higher cell viability and higher cell proliferation relative to the 10 min treatment. These results indicate that rEMS treatment for 5 min may be optimal for BM-MSC viability and proliferation, and longer treatment may be dispensable.

In order to investigate the early signaling molecules involved in rEMS-mediated cell proliferation, BM-MSCs were exposed to rEMS for 5, 10, or 15 min and cell lysates were prepared for western blot analysis of Erk1/2 activation. rEMS-mediated phosphorylation of Erk1/2 was detected in cells exposed to rEMS for 5 and 10 min (Fig. 4C); however, continuous stimulation for 15 min did not affect Erk1/2 phosphorylation, consistent with the effects on cell viability and proliferation.

Next, the present study tested the sustainability of rEMS-mediated phosphorylation of Erk1/2. When the cells were subjected to rEMS for 5 min and then allowed to sit untreated for 10 min, Erk1/2 phosphorylation returned to basal levels, similar to that of continuous 15 min rEMS treatment (Fig. 4D). Accordingly, rEMS-mediated Erk1/2 activation peaked at 5 min of treatment and spontaneously regressed to the basal level at 15 min, even when rEMS continued for 15 min. This indicates that rEMS-induced Erk1/2 activation occurred within 5 min after the initiation of rEMS treatment.

To explore the upstream signaling of rEMS-induced Erk1/2 activation, PD98059, an inhibitor of MEK, was applied before rEMS treatment (Fig. 4E). The addition of PD98059 blocked rEMS-induced phosphorylation of Erk1/2, suggesting that phosphorylation of Erk1/2 due to rEMS treatment is mediated by the MEK-Erk1/2 signaling pathway.

Previously, it was reported that rEMS treatment increased endothelial nitric oxide synthase (NOS) expression (2,11) and NO is known to induce cell proliferation through diverse signaling pathways including activation of Erk1/2 or protein kinase C (6,14). To explore the early signals mediated by rEMS treatment, whether rEMS stimulated NO production in BM-MSCs or not was investigated. As shown in Fig. 4F, rEMS treatment promoted NO production within 5 min after rEMS treatment, which was not further increased with increasing rEMS treatment times. To investigate the role of rEMS-induced NO production in Erk activation, either the carboxy-PTIO (NO scavenger) or the L-NMMA (NOS inhibitor) was used to pretreat the BM-MSCs before rEMS treatment (Fig. 4G). Blockage of NO production through carboxy-PTIO or

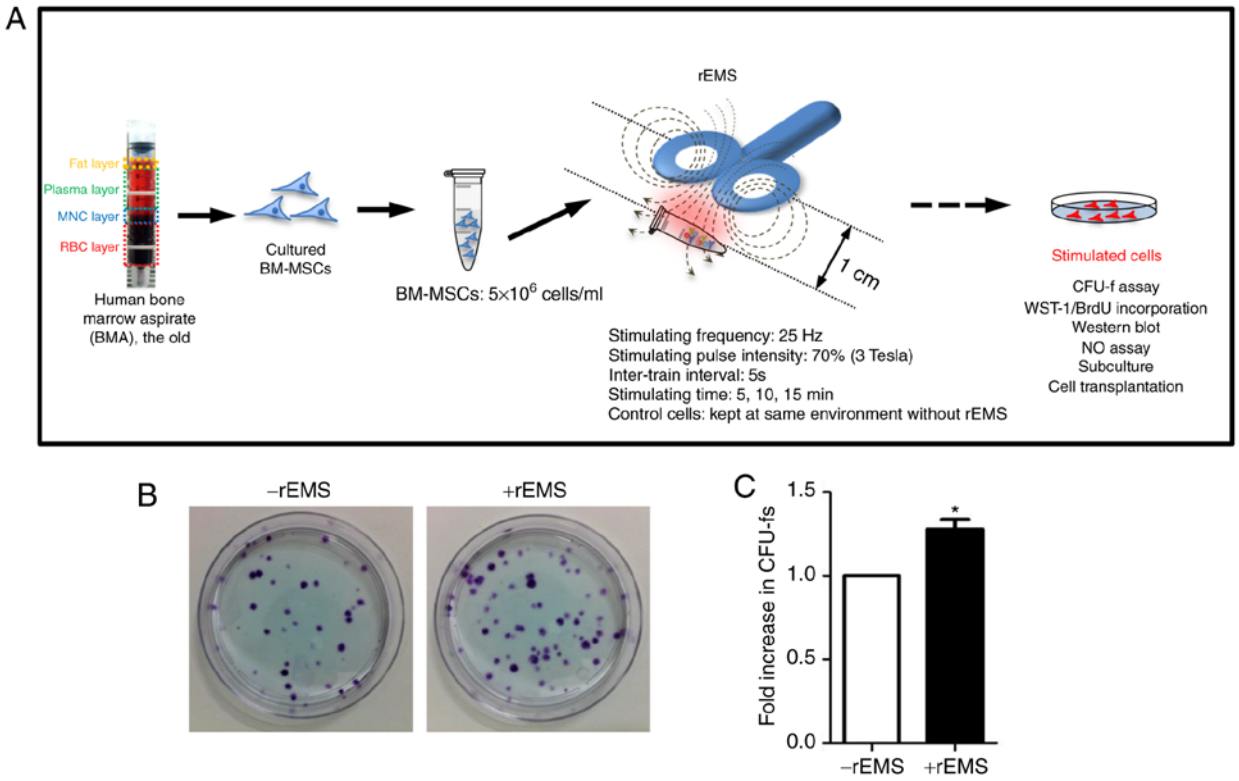

Figure 3. Effect of rEMS on colony-forming efficiency of BM-MSCs from old patients. (A) Experimental scheme of rEMS treatment and subsequent analysis. (B) MSCs from the old patients were stimulated with rEMS and plated at 37˚C in 5% CO_2. After culture for 10 days, the colonies were fixed and stained with crystal violet. Untreated MSCs were used as the control. Representative colonies from the untreated control (-rEMS) and the rEMS treatment (+rEMS) groups are shown. (C) Numbers of colonies in the rEMS treatment group and the untreated control were determined. *P<0.05 vs. -rEMS group. n=20 Data represents the mean ± standard deviation; one-way analysis of variance with Tukey's post hoc test. BM-MSCs, bone marrow-mesenchymal stem cells; CFU-f, colony forming units; rEMS, repetitive electromagnetic stimulation; RBC, red blood cell; NO, nitric oxide; WST-1, water-soluble tetrazolium salt-1; BrdU, bromo-2'-deoxyuridine.

L-NMMA treatment completely inhibited the rEMS-induced phosphorylation of Erk1/2.

Taken together, these results suggest that rEMS treatment induced NO production in BM-MSCs, which then stimulates Erk1/2 activation. Both NO production and Erk1/2 activation seem to be promptly induced within 5 min after rEMS treatment, which most likely leads to rEMS-induced cell proliferation.

rEMS-induced cell proliferation does not continue after cell passage. While the rEMS-induced proliferation of BM-MSCs may be beneficial as a preconditioning step for stem cell therapeutics, its sustained effects may evoke safety issues *in vivo*. To examine the duration of rEMS effects, BM-MSCs were subjected to rEMS treatment for 5 min, followed by BrdU-incorporating assays to directly analyze cells after rEMS treatment and after an additional cell passage (subculture; Fig. 5A). rEMS-mediated increase in BrdU-incorporating cells were observed only in the first cell generation (P<0.01 vs. -rEMS group; +rEMS group: 28.1±3.0%) and were not found in the cells after subculture, suggesting that rEMS-accelerated cell proliferation occurred transiently and was not sustained during *ex vivo* culture. Thus, application of rEMS preconditioning shortly before cell transplantation may exert positive effects on stem cell activity, which might not arouse any safety issues post-transplantation.

rEMS augments cell survival in vivo. Considering the stimulatory effect of rEMS on cellular activity, rEMS preconditioning was anticipated to augment survival or engraftment of transplanted cells *in vivo*. To track transplanted cells *in vivo*, BM-MSCs that were treated with rEMS for 5 min and labeled with PKH26 red fluorescence dye were subcutaneously transplanted into BALB/c nude mice, along with Matrigel (Fig. 5B). At eight weeks post-transplantation, numerous host cells that were not labeled with PKH26 were infiltrated into the Matrigel. Among them, the number of rEMS-treated PKH26-labeled cells was ~53% increased compared with the untreated cells (Fig. 5C and D).

This data indicates that rEMS preconditioning can improve sustainability of transplanted cells *in vivo*. As the rEMS-induced effect on cell proliferation was not sustained in the subsequent passage of cells, a high level of PKH26-labeled cells with rEMS is expected to be shown due to increased survival of BM-MSCs and not proliferation *in vivo*.

Discussion

Stem cell therapy has potential to exert many beneficial effects against critical disease. However, the very low incidence of BM-MSCs in the BMA pool and their poor cell viability is associated with the weak efficacy *in vivo*. Thus, the age-related decrease in the stem cell pool and its activity has been a critical issue and an important deterrent for the use of stem cell therapy, especially

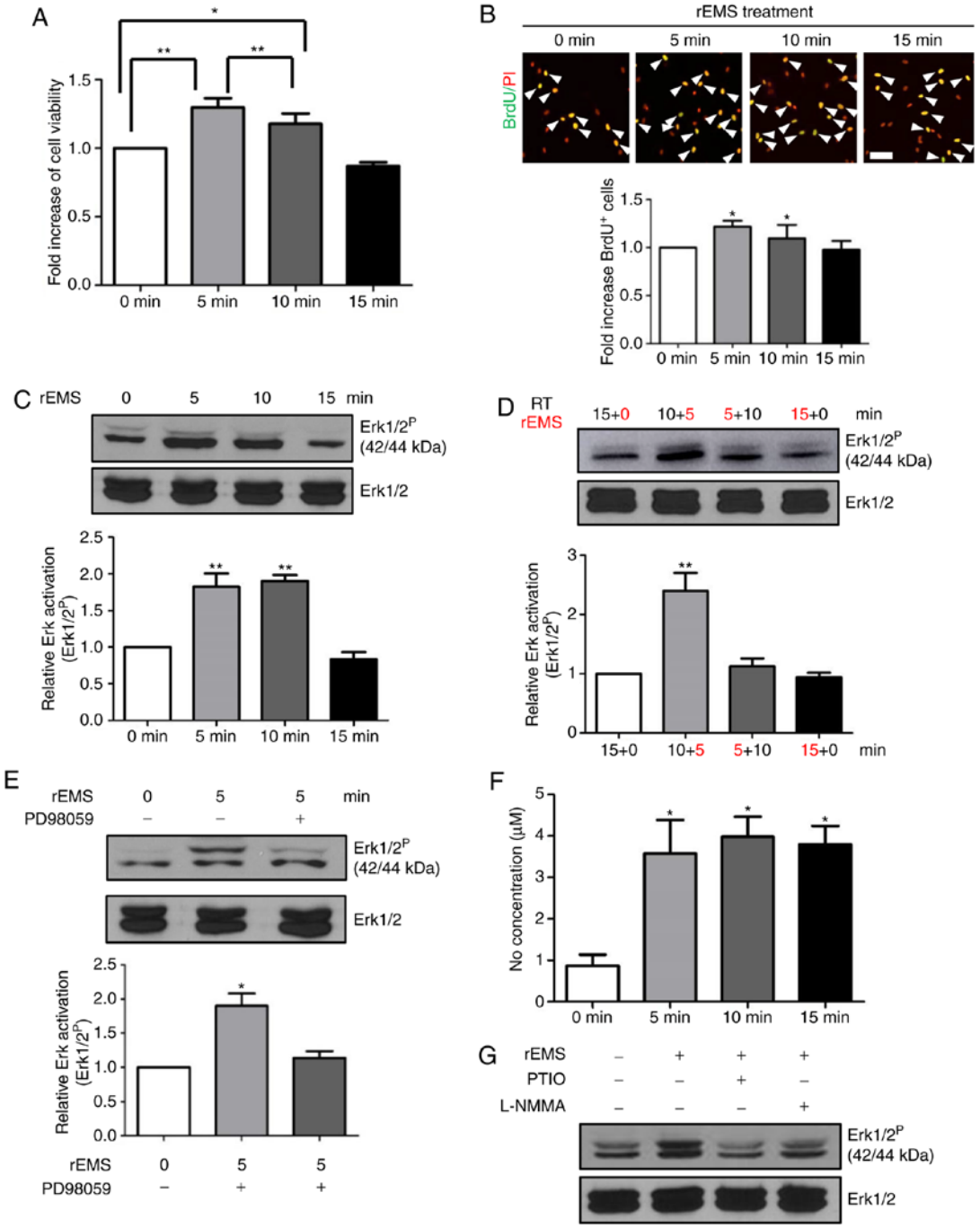

Figure 4. Effect of rEMS on BM-MSC cellular activity and intracellular signaling pathways. MSCs from the old patients were stimulated with rEMS and incubated at 37˚C in 5% CO_2. (A) At 24 h after plating, BM-MSCs were incubated with water-soluble tetrazolium salt-1 for 30 min and optical density was measured. *P<0.05 and **P<0.01 vs. 0 min. (B) BM-MSCs were incubated with BrdU for 4 h and proliferating cells were measured. Representative images of BrdU-incorporating cells (green) and PI staining for nuclei (red) of the BM-MSCs are shown. *P<0.05 vs. 0 min, n=10. White arrows: BrdU-incorporated cells. Scale bar: 100-μm. (C) rEMS-induced Erk1/2 activation was analyzed by western blotting and the quantitative analysis is shown. **P<0.01 vs. 0 min n=10. (D) Decay of rEMS-induced Erk1/2 activation is shown by western blot analysis. Duration of rEMS treatment is noted in red. **P<0.01 vs. 0 min, n=10. (E) Inhibition of rEMS-stimulated Erk1/2 activation by the mitogen-activated protein kinase/Erk kinase inhibitor PD98059. *P<0.05 vs. 0 min, n=10. (F) rEMS-stimulated NO production. *P<0.05 vs. 0 min. (G) Inhibition of rEMS-induced NO production by carboxy-PTIO NO scavenger or the L-NMMA NOS inhibitor. Data represents the mean ± standard deviation; one-way analysis of variance with Tukey's post hoc test. BM-MSCs, bone marrow-mesenchymal stem cells; CFU-f, colony forming units; rEMS, repetitive electromagnetic stimulation; L-NMMA NOS, L-NG-monomethyl arginine monoacetate nitric oxide synthase; PI, propidium iodide; Erk, extracellular signal regulated kinase; BrdU, Bromo-2'-deoxyuridine.

in older patients (21-28). The present study was designed to develop a cell preconditioning strategy to improve the activity of stem cells from BMs using the transient application of rEMS.

The present comparative study of stem cell activity from young and old patients revealed an age-related reduction in stem cell activity with a lower yield of cells. Although MSCs from the young patients were also treated with rEMS, the current goal is to enhance the activity of MSCs from the old, not the young. Therefore, after confirmation of the difference in activity of ADSC between the young and old patients, the

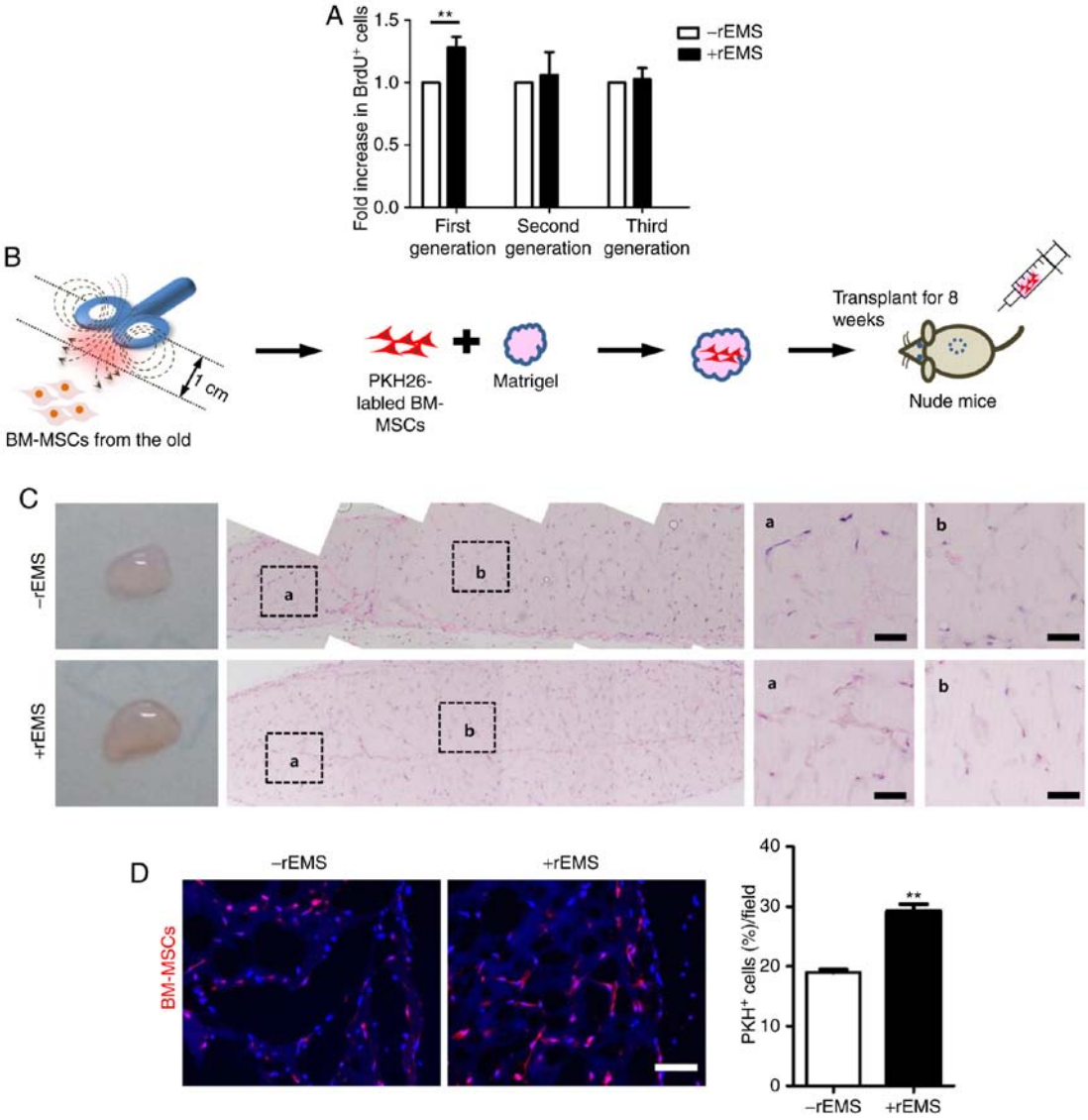

Figure 5. Evaluation of safety and the sustainability of rEMS effect on BM-MSCs. (A) BM-MSCs ($2x10^5$ cells) were stimulated with rEMS for 5 min, cultured until confluent and then sub-cultured up to the third generation. The number of BrdU-incorporating cells was counted from three independent experiments. In first generation, -rEMS group vs. +rEMS group, $^{**}P<0.01$. n=20. (B) To evaluate the effect of rEMS on the sustainability of transplanted cells, rEMS-treated BM-MSCs were labeled with PKH26, mixed with Matrigel and injected subcutaneously into nude BALB/c mice. n=6. (C) After eight weeks, the transplants were analyzed with gross view and hematoxylin and eosin staining. Scale bar: 50-μm. (D) PKH26-labeled transplanted BM-MSCs are observed as a red color and labeled cells were quantified from three independent experiments. -rEMS group vs. +rEMS group, P<0.01. n=6. Scale bar: 100-μm. Data represents the mean ± standard deviation; one-way analysis of variance with Tukey's post hoc test. $^{**}P<0.01$ vs. -rEMS group. BM-MSCs, bone marrow-mesenchymal stem cells; rEMS, repetitive electromagnetic stimulation.

present study performed all experiments with MSCs from the old patients.

This study demonstrated that rEMS treatment can increase the CFU-f and promote cell proliferation of BM-MSCs from the old patients. rEMS treatment did not influence cell-specific maker expression of BM-MSCs. This supports the hypothesis that improvement of the ability of repopulation or activity of stem cells by rEMS treatment did not alter the characteristics of BM-MSC. Moreover, considering the beneficial effects of rEMS treatment on stem cells, the differentiation potential of stem cell may be improved, which should be determined in further study.

Interestingly, rEMS treatment enhanced sustainability of transplanted BM-MSCs from the old patients without any safety issues. The intensity of PKH 26 is decreased as the cell divides. However, transplanted cells rarely proliferate *in vivo*. Thus, 50% more PKH26-labeled cells with rEMS treatment may be not due to proliferation *in vivo*. This phenomenon is inferred to be related to survival rate. PKH-26 cells with rEMS might undergo apoptosis at a reduced rate compared with PKH-26 cells without rEMS *in vivo*. rEMS treatment may be involved in cell survival though controlling cellular activity or paracrine action *in vivo* and the mechanism of cell survival (rEMS-treated cells) will be explored in the future.

Under the current experimental conditions, the rEMS-mediated positive effect seems to have peaked and was saturated within 5 min of treatment. In multiple experiments, longer rEMS treatment did not further increase NO production, Erk1/2 activation, or cell proliferation. Furthermore, continuous rEMS treatment for 15 min resulted in lower Erk

activation compared with that observed at 5 min, which is most likely due to the spontaneous deactivation of Erk achieved by the 5 min of rEMS treatment. The present study confirmed that rEMS treatment for 5 min is sufficient for BM-MSC preconditioning.

More importantly, rEMS treatment was effective in stem cells from old rather than young patients. Stem cells are chiefly transplanted to old patients with critical diseases and these patients might have a stem cell pool of low activity. Currently, allogenic transplantations of stem cells are performed in the clinic; however, depending on background disease, the use of allogenic cells is impracticable due to toxicity. Thus, the restoration of the activity of stem cells from old patients could have great potential in therapeutics and may become a safe treatment approach used clinically. The present study investigated the effect of rEMS on the stem cell pool of old patients and confirmed the possibility for application of rEMS to stem cell therapy in aged patients suffering from chronic diseases including diabetes.

Moreover, current commercial rEMS instruments, which are designed with two overlapping loops of wire in a figure-eight arrangement to focus narrowly in deep areas of the brain, are not easily modified for the electromagnetic stimulation of cell cultures. The present configuration could be a useful tool for other clinical applications, but diverse parameters such as stimulation intensity, frequency and distribution of the electric current by magnetic coils may be another important consideration in the analysis of the exact series of signaling cascades and their interplay, which needs to be further explored (29,30).

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Availability of data and materials

The datasets used in the present study are available from the corresponding authors upon reasonable request.

Authors' contributions

SN, SK, KY, YS and HH performed all experiments and interpreted data. HH and YS drafted and finalized the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee for Experimental Animals of Kyung Hee University (approval no. KHMC-IACUC-14-010).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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