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Therapeutic Effect of Human Mesenchymal Stem Cell-Conditioned Medium on Erectile Dysfunction

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Purpose: Owing to the safety and cost effectiveness of conditioned medium (CM), its therapeutic effects have attracted significant attention from many researchers. To date, numerous studies have been conducted on CM; however, little has been done with regard to erectile dysfunction (ED). In this research, the potential of human mesenchymal stem cell-derived CM (MSC-CM) for the treatment of ED was investigated.

Materials and Methods: A high concentration of MSC-CM was prepared through 3D spheroid culturing with bone marrow-derived MSCs and cut-off filtering. The composition of CM was analyzed using biochemical assays, and the effect of the preparation process on the quality of CM was investigated. The therapeutic effects of MSC-CM were evaluated through animal studies using a cavernous nerve (CN) injury rat model.

Results: 3D spheroid culturing afforded a 278-fold increase in the total protein content of CM, as compared to that from 2D cultures; the protein concentration increased by 19 times on increasing the centrifugation time for cut-off filtering. Biochemical assays indicated that the CM contains various types of angiogenic, neurotrophic, and anti-inflammatory factors. Histological assay results showed that MSC-CM has angio- and neuro-trophic effect in a CN injury rat model *in vivo*, and these therapeutic effects appear in a dose-dependent manner.

Conclusions: The experimental results confirmed the therapeutic effect of MSC-CM in healing damaged cavernosal tissue and restoring erectile function. These results successfully demonstrated that MSC-CM has significant potential for the treatment of ED.

Keywords: Angiotrophic effect; Conditioned medium; Erectile dysfunction; Mesenchymal stem cell; Neurotrophic effect

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INTRODUCTION

Erectile dysfunction (ED) is a sexual disorder, defined as problems with erectile function and its maintenance [1]. There are various causes of ED, with damage in blood flow and nerves being the typical ones [2,3]. Therefore, to restore erectile function, a treatment method that is effective in angiogenesis, nerve protection, and regeneration is required. Various types of medicines have been applied for the treatment of ED [4,5]. Among them, phosphodiesterase type-5 inhibitors are widely used in clinical practice. However, it is known that the therapeutic effect of these inhibitors is low because of side effects, such as smooth muscle relaxation and collagen accumulation [6]. Recently, stem cell technology has attracted great attention as a viable option for overcoming the difficulties in the treatment of ED [7,8]. However, stem cell therapy requires the infusion of tens of millions of cells due to the low rate of engraftment *in vivo* and has the potential to develop into tumors [9-11]. In addition, a long preparation time and high costs are associated with the storage and culture of cells, and there is a possibility of an immune response [12,13].

To compensate for the shortcomings of stem cell therapy, development of conditioned medium (CM)-based therapy using trophic factors and cytokines secreted from cells has increasingly attracted the attention of researchers [14-16]. Stem cell-based CM therapy has the advantage of using the paracrine action of stem cells while excluding the possibility of tumor development. Most importantly, CM therapy has low immune response, and provides an off-the-shelf solution because CM can be stored after production [14,16]. Various studies have been conducted on the regenerative effect of CM therapy [17,18]. However, little has been done on ED treatment. Sun et al [19] reported the therapeutic effects of human bone marrow mesenchymal stem cell-derived CM (hBM MSC-CM) on diabetes-derived rat ED model, and also showed similar treatment effects with cell therapy. However, they only examined the neurotrophic effects of MSC-CM but not the effects on endothelium dysfunction and fibrosis, which are the general phenomena in ED.

In this research, the therapeutic effect of hBM MSC-CM on ED was investigated. MSC-CM was harvested from MSC spheroids, and quantitative analysis was performed to identify factors, particularly angiotrophic and neurotrophic factors, in the CM. Finally, the prepared CM was applied to a rat ED model to investigate its therapeutic effect in terms of endothelial dysfunction, fibrosis, and neurotrophic effect.

MATERIALS AND METHODS

1. Cell culture and preparation of conditioned medium

hBM MSCs (under 7 passages; Lonza, Basel, Switzerland) were cultured with low glucose Dulbecco's modified Eagle's medium (DMEM; Welgene, Gyeongsan, Korea) supplemented with 10% fetal bovine serum (FBS; Capricorn, Ebsdorfergrund, Germany) and 1% penicillin/streptomycin (Capricorn). Green fluorescent protein-tagged human umbilical vein endothelial cells (under 7 passages, GFP-HUVEC; Angio-proteomie, Boston, MA, USA) were cultured in Endothelial Growth Medium-2 (Lonza).

CM was prepared using MSC spheroids. A 12-series micro-mold (The 3D Petri Dish®; Merck KGaA, Darmstadt, Germany) was used to prepare microwells following manufacturer's instructions. After plating MSCs, the microwells were incubated at 37°C, 5% CO_2 for 3 days to induce cell spheroids. After aspirating the medium, 2 mL of serum-free DMEM added, and CM was collected after 48 hours of incubation. For two-dimensional (2D) culture group, MSCs in 6-well plates were fed 2 mL of serum-free DMEM at 70% to 80% confluence and incubated for 48 hours to obtain CM. The collected CM samples were concentrated using 3-kDa cut-off centrifugal filter units (Merck KGaA) following the manufacturer's instructions. The Bradford assay reagent (Sigma Aldrich Co., St. Louis, MO, USA) was used to measure protein concentration in the CM. The CM was stored at -80°C until use. As needed, the CM was diluted with serum-free medium. Three groups (CM 100%, CM 50%, and CM 10%) with different concentrations of CM were prepared to investigate the concentration effect.

2. Cell viability assay

Cell viability was investigated using a live/dead assay kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. After staining, the cells were observed with fluorescence microscope (Leica DM2500; Leica Microsystems AG, Wetzlar, Germany).

3. Biochemical assay

Proteome Profiler Human XL Cytokine Array Kit (R&D Systems, Minneapolis, MN, USA) was used to identify 105 cytokines in the CM following the manufacturer's instructions. The concentrations of angiogenin (ANG), vascular endothelial growth factor (VEGF), brain derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and nerve growth factor (NGF) in the CM were measured using commercial ELISA kits (ANG, VEGF, and BDNF: R&D Systems; GDNF and NGF: Raybiotech, Norcross, GA, USA) following the manufacturer's instructions.

4. Tube formation assay with GFP-HUVEC

A 24-well plate was coated with 289 μL of growth factor-reduced Matrigel (Corning, NY, USA) of 10 mg/mL. GFP-HUVECs (1.5×10^5) cells were suspended in 300 μL hBM MSC-CM and seeded on the coated well plate. After incubation at 37℃ for 4 hours, tube formations of GFP-HUVEC were observed with a fluorescence microscope (Leica DM2500; Leica Microsystems AG). After imaging, the number of nodes and meshes was counted for quantification by using ImageJ (National Institutes of Health, Bethesda, MD, USA).

5. Animal study

Cavernous nerve (CN) injury model was prepared using Sprague-Dawley rats (9 weeks old; Orient Bio Inc., Seongnam, Korea) following the procedure described previously [20]. Phosphate buffered saline (PBS) was used for the preparation of control group for animal study because vehicle type has no significant effect on erectile function [8,20,21]. CM or PBS (control group) (50 μL) was intracavernously injected into the corpus cavernosum using 30 G needle. Immediately prior to injection, the penile base was compressed using a bulldog clamp to halt drainage *via* dorsal vein. The compression was released 1 minute after the injection. Through this procedure, the following five groups were prepared: sham, control, CM 10%, CM 50%, and CM 100%. In the sham group, only laparotomy was performed without CN injury. After 4 weeks, the rats were sacrificed after assessing their erectile function. All procedures for animal studies were approved by the Institutional Animal Care and Use Committee of Asan Medical Center, Korea (IACUC protocol number: 2018-13-155).

6. Measurement of erectile function

The erectile function was evaluated as described previously [20]. After 4 weeks of CM injection, 200 μL of tiletamine was injected intramuscularly to anesthetize all the rats. The right carotid artery was cannulated using heparinized 24 G silastic cannula. A continuous pressure transducer was connected to the cannula to measure mean arterial pressure (MAP). The penile corpus cavernosa and the major pelvic ganglion (MPG), posterolateral to the prostate, were exposed. For measurement of intracavernous pressure (ICP), a heparinized 23 G needle was inserted into the corpus cavernosum. Electrical stimulation (5 V at 10 Hz for 1 min) was applied to the MPG using bipolar hook electrode, and the change in ICP was measured. Electrical stimulation was performed at least twice, and the interval between stimulations was more than 10 minutes. To compare erectile function among the groups, the total ICP was divided by the MAP and designated as ICPtotal/MAP.

7. Masson's trichrome staining

Masson's trichrome staining was performed by using dibutyl phthalate xylene (Sigma-Aldrich Co.) as previously described [20].

8. Immunostaining

As described in detail previously [20], samples were fixed in 4% paraformaldehyde and blocked with normal goat serum. For smooth muscle and the endothelial content staining, anti-α smooth muscle actin (αSMA) (1:400; Sigma Aldrich Co.), anti-endothelial nitric oxide synthase (eNOS) (1:100; Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and anti-von Willebrand factor (vWF) (1:400; Abcam, Cambridge, UK) were used. For neuronal staining, anti-neuronal nitric-oxide synthase (nNOS) (1:100; Novus Biologicals, Centennial, CO, USA) was used. The nucleus was stained using 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Goat anti-mouse and goat anti-rabbit (1:400; Thermo Fisher Scientific) were used as secondary antibodies.

9. Statistical analysis

The data are expressed as mean±standard error of the mean. Statistical comparisons between experimental groups were analyzed using one-way analysis of variance followed by the Tukey's honest significant difference test for post hoc comparisons or nonpara-

metric Kruskal–Wallis analysis. A p-value <0.05 was considered as statistically significant.

RESULTS

1. Viability of hBM MSC spheroids and cytokine secretion

Cell spheroids, with diameters of 150, 300, and 450 μm, were prepared and the cell viability was investigated by live/dead staining (Fig. 1A). Overall, most of the cells showed good survival in all groups. However, the proportion of dead cells in the spheroids was slightly increased as the spheroid size increased. The amount of cytokines secreted from the spheroids was measured using Bradford assay. Cytokine secretion was greatly improved through spheroid culture (Fig. 1B). The prepared cell spheroids showed an increase of about 19 to 264 times in cytokine secretion compared to the secretion by 2D cultured cells. Similar to the results of the viability test, cytokine secretion decreased with increasing size of spheroids. The spheroids, 450 μm in diameter, showed about 14.1-times less cytokine secretion than the 150-μm diameter spheroids. Based on these results, 150-μm cell spheroids were used for the preparation of CM in the following experiments.

2. Effect of centrifugation time on the concentrations of growth factors

The effect of centrifugation using a cut-off filter on CM preparation was investigated by measuring the concentrations of growth factors. The Bradford assay of the prepared CMs showed that the total protein concentration increased exponentially with increasing centrifugation time (Fig. 2A). The total protein content increased by approximately 19-fold upon centrifugation for 110 minutes compared to the content when centrifugation was done for 30 minutes. Although the average protein content increased when centrifugation was done for 110 minutes, the difference was not significant when compared to the content obtained after centrifugation for 90 minutes.

The concentrations of cytokines in the CMs prepared by 30 and 110 minutes of centrifugation were determined using the Human XL Cytokine Array kit containing 105 antibodies. Positive blotting was observed for most antibodies used in the analysis (Fig. 2B). Angiotrophic (ANGPT-1, ANG, and VEGF) and neurotrophic (BDNF and bFGF) factors were identified in the CM, and various anti-inflammatory cytokines, such as TNF-α, IL-1ra, and IL-4, were also found. Quantitative data on the concentrations of the cytokines were obtained by ELISA using the blotting results, and fold changes in cytokine concentrations between the conditions of 30 and 110 minutes centrifugation times were calculated (Fig. 2C). Among the measured cytokines, the concentrations of angiotrophic factors were the highest. The concentrations of VEGF and ANG changed by about 7- and 4-times, respectively. Among the neurotrophic factors, the concentration of bFGF showed a 3-fold change, and for other factors, about 2-fold changes were observed. On the contrary, anti-inflammatory cytokines showed less than 2-fold difference in all the analyzed components.

Finally, the concentration of angiotrophic and neurotrophic factors contained in hBM MSC-CM (90 minutes centrifugation condition) was measured using an ELI-

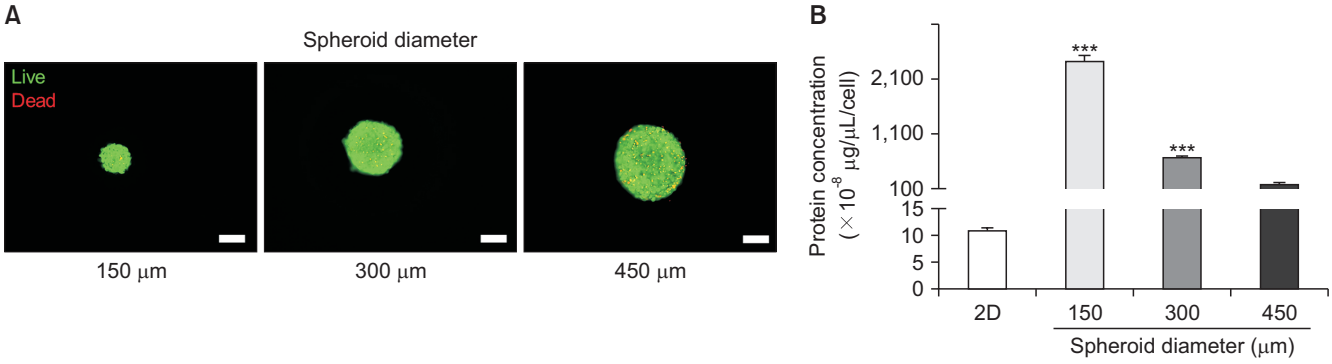

Fig. 1. Effect of spheroid diameter on cell viability and protein concentration in CM. (A) Fluorescence microscopy images of mesenchymal stem cell spheroids, having different diameters, stained with calcein-AM (green, live cells) and ethidium-1 (red, dead cells) (scale bars, 150 μm). (B) Concentrations of total proteins in CM. Data are shown as mean±standard error of the mean (n=3). ***p<0.001 compared to the 2D group. CM: conditioned medium, 2D: two-dimensional.

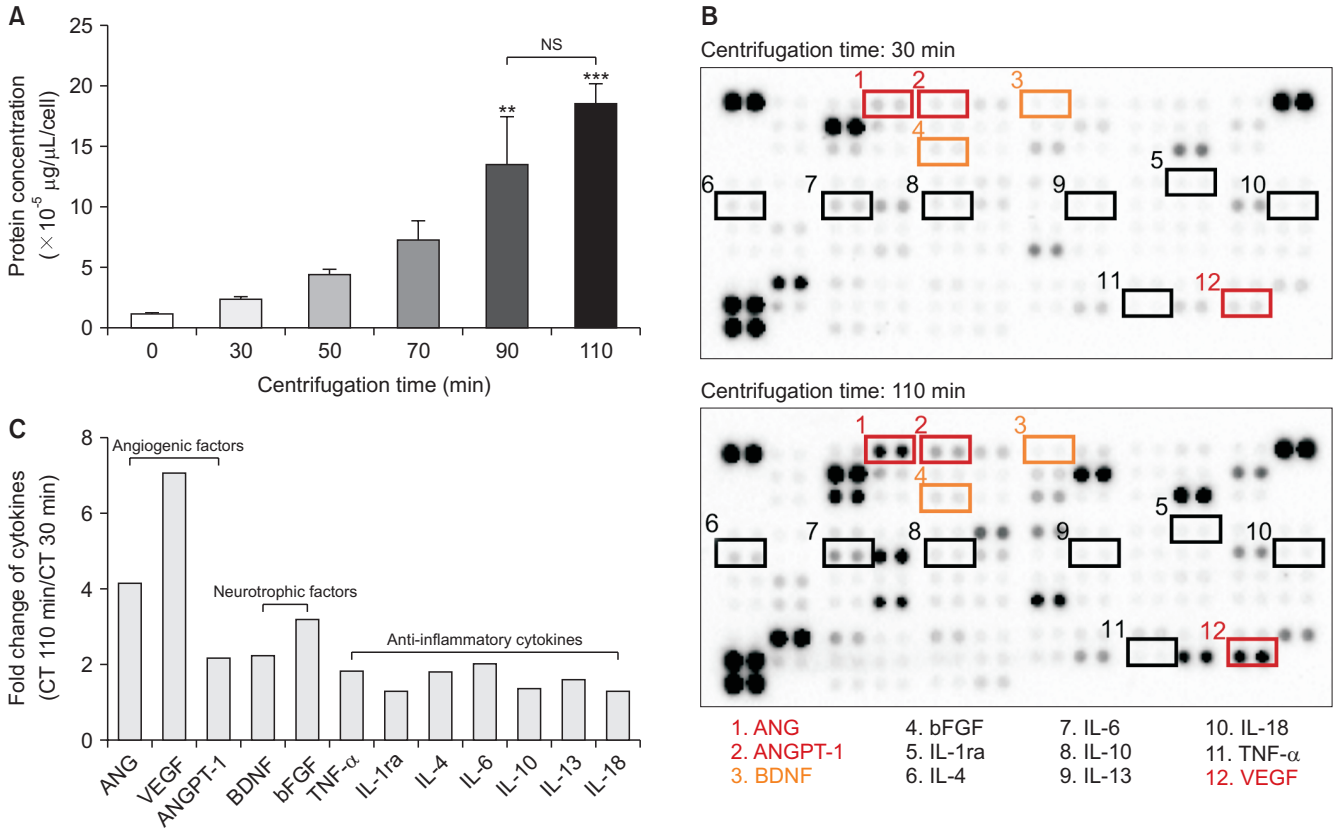

Fig. 2. Effects of CT on the concentration of cytokines in mesenchymal stem cell-derived conditioned medium (MSC-CM). (A) Total protein concentration in MSC-CM with changes in the CT. Data are shown as mean±standard error of the mean (n=3). **p<0.01 and ***p<0.001 compared to the control group. (B) Assay results for Human Cytokine Antibody Array containing 105 antibodies obtained with CM prepared using CTs of 30 and 110 minutes. The rectangles represent cytokines associated with angiogenic (red) and neurotrophic (orange) factors and anti-inflammatory cytokines (black). (C) Fold changes in the amounts of individual cytokines obtained with CTs of 110 and 30 minutes. The fold change was calculated using optical density values measured from the assay results (B). NS: no significance, CT: centrifugation time.

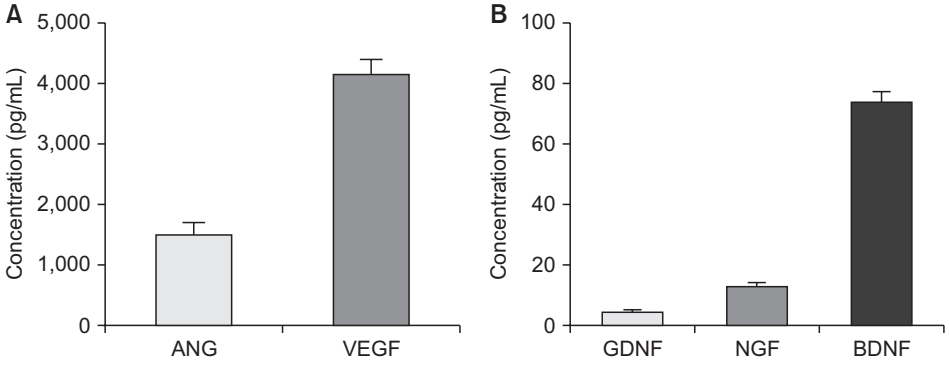

Fig. 3. Concentration of (A) angiogenic and (B) neurotrophic factors in mesenchymal stem cell conditioned medium (CM). The CM was prepared using 90 minutes centrifugation time, and the concentration of the factors was measured using ELISA. All data are shown as mean±standard error of the mean (n=6).

SA kit (Fig. 3). The concentrations of ANG and VEFG in the CM were 1,503.4±191.5 pg/mL and 3,811±352 pg/mL, respectively. The concentrations of neurotrophic factors NGF, GDNF, and BDNF, were found to be 4.3±0.036 pg/mL, 12.6±3.3 pg/mL, and 74.7±1.8 pg/mL, respectively.

3. *In vitro* assay of the effect of hBM MSC-CM on angiogenesis

A tube formation assay using GFP-HUVEC was conducted to evaluate the effect of hBM MSC-CM on angiogenesis. After treatment of endothelial cell-laden Matrigel with 25% to 50% CM, tube formation was observed under a fluorescence microscope. DMEM me-

dium without CM—rmfjDMEM only group—was used as a negative control. It was qualitatively confirmed that as the concentration of CM increased, tube formation was activated (Fig. 4A). This tendency could be observed more clearly in the quantification results for the number of nodes and meshes and the total length of tube (Fig. 4B-4D). Overall, all the calculated values increased as the CM concentration increased. The CM 100% group showed about 14-times increase in the tube length compared to the DMEM only group, and the number of meshes and nodes was 25- and 15-times higher, respectively. Statistical significance was also observed for most of the differences except for a few. Based on these results, it was confirmed that hBM-MSC CM has a concentration-dependent effect on angiogenesis.

4. Evaluation of erectile functions in rats injected with the CM

After the injection of hBM MSC-CM into the CN injured rats, their erectile functions were investigated by measuring ICPtotal and MAP during MPG stimulation (Fig. 5). It was observed that the ICPtotal/MAP value increased with the CM concentration. The CM 100% and 50% groups showed functionalities of approximately 73% and 66%, respectively, as compared to the positive control (sham group). Statistical significances between the groups and the negative control were also observed. The negative control only exhibited a functionality of approximately 40%.

5. Effect of hBM MSC-CM on fibrosis

After getting cavernosal tissue from the penis of the CN injured rats, the tissue was stained with Masson's

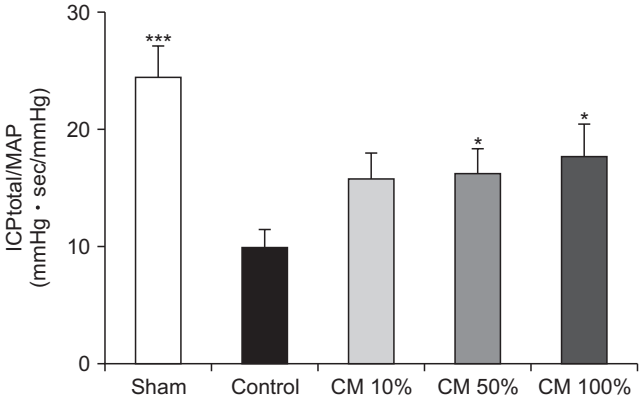

Fig. 5. *In vivo* evaluation of erectile function. After injecting 10% to 100% conditioned medium (CM) into rats with erectile dysfunction, the ratio of intracavernous pressure (ICP) to mean arterial pressure (MAP) was measured to evaluate the erectile function. In the control group, phosphate-buffered saline was injected into the rats. Data are shown as mean±standard error of the mean (n=20 for each group). *p<0.05 and ***p<0.001 compared to the control group.

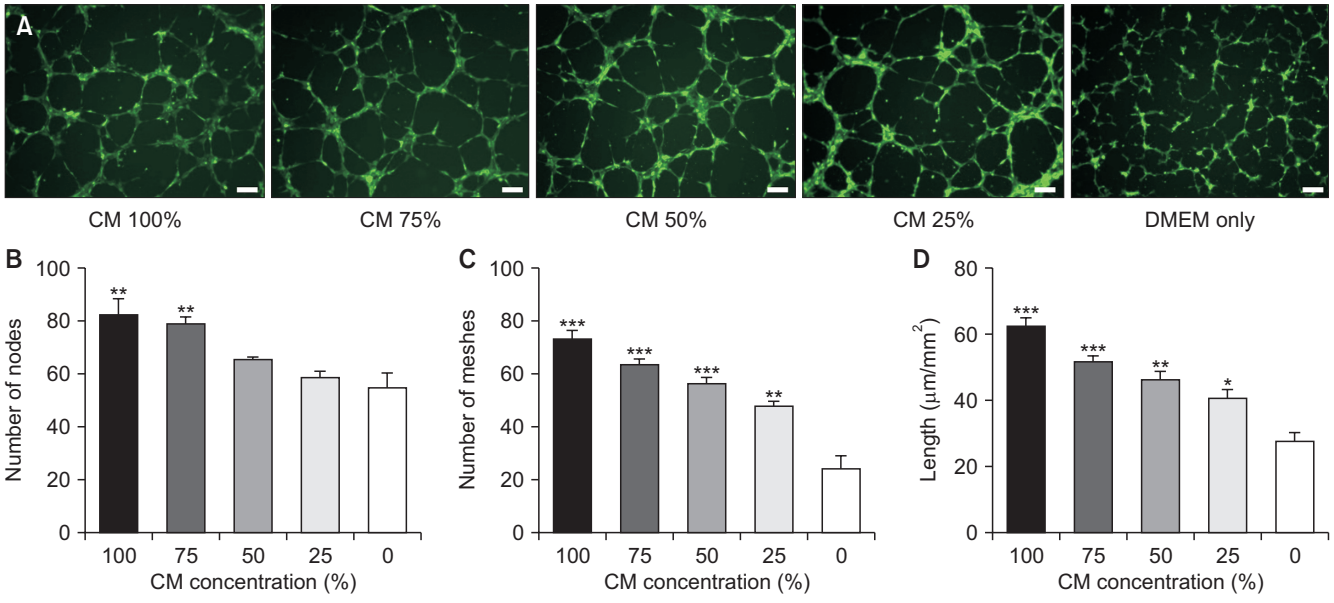

Fig. 4. *In vitro* endothelial cell tube formation assay using conditioned medium (CM). (A) Representative fluorescence images showing the results of endothelial cell tube formation assay at different concentrations of the CM (scale bars, 100 μm). After embedding into the Matrigel, GFP-labeled human umbilical vein endothelial cells were incubated for 4 hours. (B-D) Results of quantitative analysis based on the fluorescence images for the number of (B) nodes and (C) meshes and (D) total tube length. Data are shown as mean±standard error of the mean (n=4). *p<0.05, **p<0.01, and ***p<0.001 compared to DMEM only group, respectively.

trichrome and αSMA to investigate fibrosis. It was observed that with the increase in CM concentration, the collagen density decreased, and the proportion of smooth muscle increased (Fig. 6A). This tendency was observed more clearly by quantifying. The ratio of smooth muscle area to collagen area increased with increasing CM concentration (Fig. 6B). Statistical significance was also observed. Immunofluorescence staining with αSMA also showed similar results (Fig. 6C, 6D). As the concentration of injected CM increased, smooth muscle content increased. However, only the CM 100% group showed statistical significance with respect to the control group. Based on these results, it was confirmed that CM injection can significantly alleviate the progression of fibrosis and reduction in the smooth muscle content of cavernosal tissue caused by CN damage, and the effect is proportional to the CM concentration.

6. *In vivo* angiotrophic effect of hBM-MSC CM

Immunohistochemical staining for eNOS and vWF was performed on the cavernosal tissue to investigate *in vivo* angiotrophic effect of BM-MSC CM (Fig. 7A, 7B). The higher the CM concentration, the more eNOS- and vWF-positive areas and blood vessel structures were observed. The CM 100% group showed a morphology very similar to that of the sham group. In contrast, in the control group, serious damage was caused to the blood vessels of the cavernosal tissue by CN injury. This tendency was more clearly observed in the quantification results based on the immunostaining results (Fig. 7C, 7D). As the concentration of CM increased, the proportion of eNOS- and vWF-positive regions continued to increase, and the staining was the least in the control group. These differences were statistically significant. Based on these results, it was confirmed that BM-MSC CM is effective in preserving the vasculature of cavernosal tissue in the case of CN injuries, and this effect is dose dependent.

7. *In vivo* neurotrophic effect of hBM-MSC CM

nNOS immunostaining was performed on dorsal penile nerve of CN-injured rats to investigate *in vivo* neurotrophic effects of CM (Fig. 8). Fluorescence images showed a significantly increased nNOS-positive area in the CM 100% group compared to that in the CM 10% and 50% groups (Fig. 8A). Moreover, severe damage to the dorsal penile nerve was observed in the

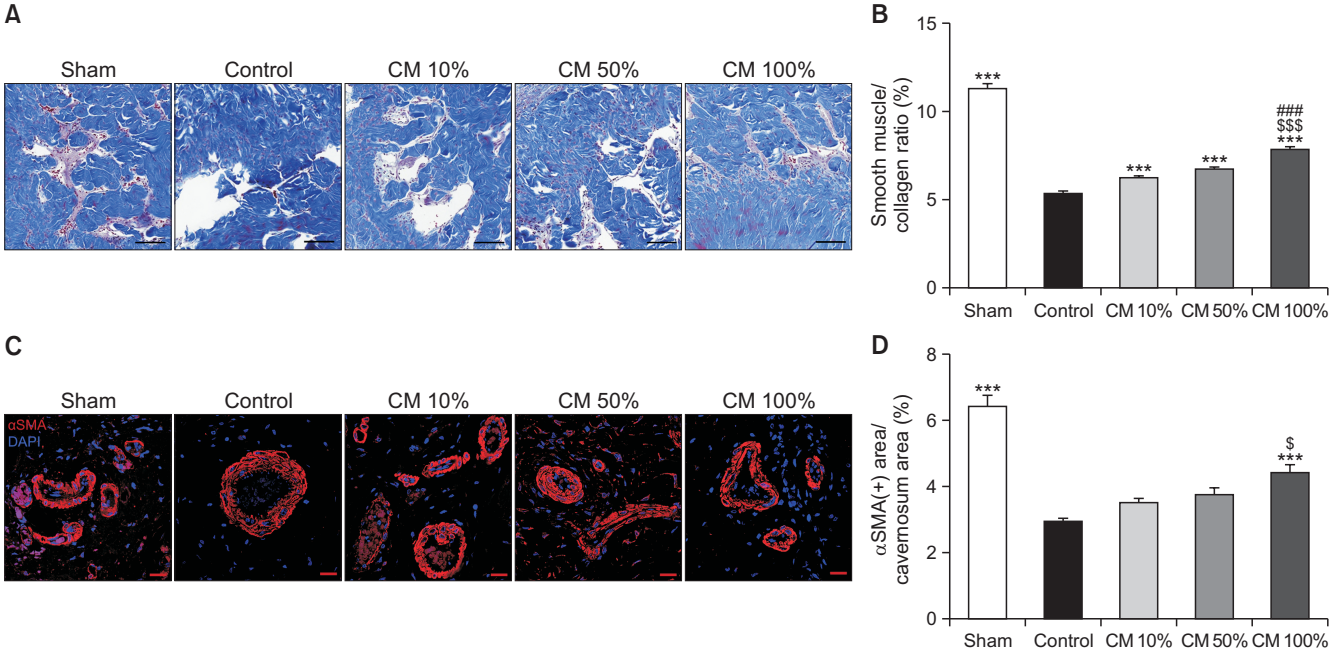

Fig. 6. Masson's trichrome and alpha smooth muscle actin (αSMA) staining. (A) Microscopic and (C) fluorescence images showing the results of Masson's trichrome and αSMA staining of corpus cavernosum tissues of mice injected with 10% to 100% conditioned medium (CM), respectively (scale bars, 100 μm). The (B) ratios of smooth muscle area to collagen area and (D) αSMA positive-stained area to cavernosum tissue area measured through processing of the images obtained in the (A) Masson's trichrome and (C) αSMA staining. In the control group, phosphate-buffered saline was injected into the rats. Data are shown as mean±standard error of the mean (n=40). ***p<0.001 compared to the control group; $^{\$}$p<0.05 and $^{\$\$\$}$p<0.001 compared to CM10% group; $^{\#\#\#}$p<0.001 compared to CM50% group.

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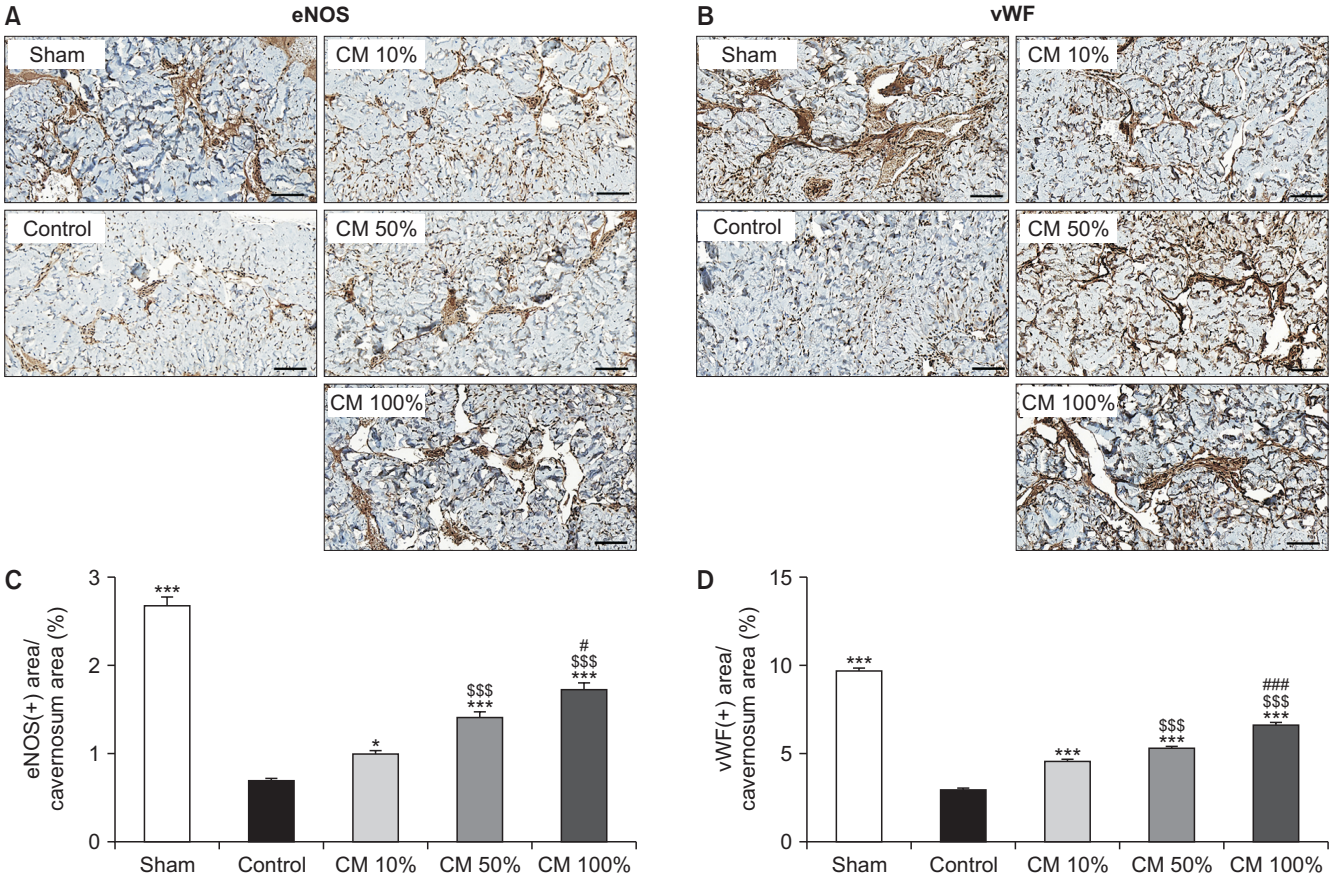

Fig. 7. Endothelial nitric oxide synthase (eNOS) and von Willebrand factor (vWF) immunostaining. Microscopic images of (A) eNOS and (B) vWF immunostaining of corpus cavernosum tissues of mice injected with 10% to 100% conditioned medium (CM) (scale bars, 100 μm). The ratios of (C) eNOS- and (D) vWF-stained areas to cavernosum areas measured through the processing of the microscopic images (A) and (B). Data are shown as mean±standard error of the mean (n=40). *p<0.05 and ***p<0.001 compared to the control group; $^{\$\$\$}$p<0.001 compared to CM10%; $^{\#}$p<0.05 and $^{\#\#\#}$p<0.001 compared to CM50%.

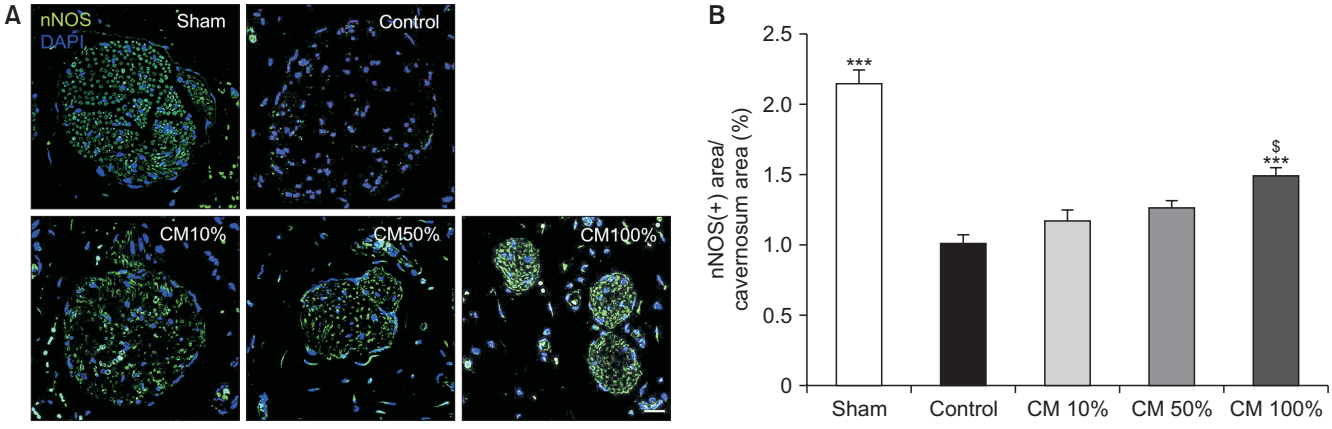

Fig. 8. Neuronal nitric oxide synthase (nNOS) immunostaining. (A) Fluorescence microscopic images of nNOS immunostaining results of corpus cavernosum tissues of rats injected with 10% to 100% conditioned medium (CM) (scale bars, 100 μm). (B) The ratios of nNOS-stained areas to cavernosum areas measured through the processing of the microscopic image (A). Data are shown as mean±standard error of the mean (n=40). ***p<0.001 compared to the control group; $^{\$}$p<0.05 compared to CM10% group.

control group. Quantitative analysis based on the immunofluorescence images showed that proportion of nNOS-positive area increased with increasing CM concentration. Statistical significance was also observed in the CM 100% group, but not in other groups. Based on these results, it was confirmed that the BM-MSC CM

has a therapeutic effect in preserving the dorsal penile nerve in the case of CN injuries *in vivo*.

DISCUSSION

Numerous studies on the development of stem cell therapy for the treatment of ED have been performed considering the potential for tissue regeneration [7,12,13]. However, many challenges in terms of cost and safety associated with stem cell therapy need to be surmounted in view of the low rate of *in vivo* engraftment and the potential for immune response and tumor formation [9-14]. In this regard, CM therapy, using stem cell secretomes, has attracted great attention from many researchers owing to its safety and cost-effectiveness compared to stem cell therapy [14-16]. In this research, the angiotrophic and neurotrophic effects of BM-MSC CM were investigated to evaluate its potential for the treatment of ED.

hBM-MSC CM had a therapeutic effect on ED, and this effect was improved in a CM-concentration dependent manner. First, it was confirmed that hBM-MSC CM had an angiotrophic effect in CN injured rats. In an *in vitro* tube formation assay using HUVEC, tube formation was activated with increasing CM concentration (Fig. 4). Similar results were observed in an *in vivo* experiment using the CN injury rat model. It was observed that the CM injected group had significantly higher endothelium content than the negative group (Fig. 7). The neurotrophic and anti-apoptosis effects of CM were also confirmed. In the CM injected group, the nerve and smooth muscle content was substantially higher than in the negative control group (Fig. 6, 8). In addition, the erectile function of rats was significantly improved by about 1.8 times upon CM 100% injection in CN injury model compared to the function in non-injected rats (Fig. 5). Above all, the therapeutic effect improved in a CM concentration-dependent manner. Interestingly, these results are highly similar to those of stem cell injection in a CN injury model [20,22]. MSC secretes various types of trophic factors that can be responsible for the therapeutic effects [14,17-19,23,24]. Sun et al [19] reported an *in vivo* neurotrophic effect of MSC-CM and showed that the CM contains various types of neurotrophic factors, such as BDNF and NGF. Santos et al [17] showed that VEGF and ANG in MSC-CM have angiotrophic effects in the wound healing process. In this research, it was confirmed that the prepared hBM MSC-CM possesses various factors, such as angiotrophic factors, neurotrophic factors, and anti-inflammatory cytokines. The trophic factors in CM are assumed to be effective in healing damaged cavernosal tissue and in restoring the erectile function in CN-injured rats.

CONCLUSIONS

In this research, the effect of hBM MSC-CM in the treatment of ED was investigated. CM with high protein concentration was prepared through 3D spheroid culturing and cut-off filtering. It was also verified that the MSC-CM contains various types of angiogenic, neurotrophic, and anti-inflammatory factors. Finally, the *in vivo* angio- and neurotrophic effects of the CM were successfully confirmed using a CN injury rat model. Interestingly, the effects were dose-dependent. This study confirms the high potential of hBM MSC-CM therapy for the treatment of ED. Based on these results, further studies will be conducted on technologies to improve the therapeutic effect of the hBM MSC-CM.

Conflict of Interest

The authors have nothing to disclose.

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Author Contribution

Conceptualization: KHM, HWK. Data curation: SGK, DY, KK, JA, YSK, MJJ. Formal analysis: DY, KHM, HWK. Methodology: SGK, DY, KK, JA, YSK, MJJ. Supervision: DY, KHM, HWK. Visualization: SGK, DY. Writing – original draft: SGK, DY, HWK. Writing – review & editing: KHM, HWK.

Data Sharing Statement

The data analyzed for this study have been deposited

in HARVARD Dataverse and are available at https://doi.org/10.7910/DVN/CHSNJQ.

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