Master's Thesis

Angiogenic and Neurotrophic Effects of Human Bone Marrow Mesenchymal Stem Cell-Derived Conditioned Medium for Erectile Dysfunction

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Approved by

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ABSTRACT

A three-dimensional spheroid culture system to harvest conditioned medium (CM) from human bone marrow-derived mesenchymal stem cells (hBM-MSCs) with high efficiency were introduced and investigated the therapeutic effects of hBM-MSC-CM on erectile dysfunction (ED). In order to harvest CM with higher protein concentration than CM harvested by conventional method in 2D culture system, a variable parameter study considering the culture conditions such as spheroid diameter and centrifugation time was performed. As a result, it was confirmed that CM harvested from 3D spheroid culture system had much more proteins than those cultured in 2D environment. In addition, the total protein concentration in CM increased in proportion to the centrifugation time using a centrifugal filter. Additionally, ELISA and secretome assay showed that CM contained growth factors that help in angiogenesis such as angiogenin (ANG) and vascular endothelial growth factor (VEGF), and in nerve regeneration like glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF). Also, it was identified that angiogenic effects of CM contributing to tube, node and mesh formation of endothelial cells was proportional to the concentration of CM in vitro tube formation assay. Then, histomorphometric studies such as immunohistochemistry and immunofluorescence were performed using the cavernous nerve (CN) injury rat model. In result, histomorphometric analysis showed that neural regeneration and vascular regeneration increased along with CM concentration. To sum up, we established an efficient method to obtain hBM-MSC-CM containing high concentration of growth factors, which is expected to be one of the promising methods of regenerative medicine for treating ED disease.

Keywords: Human bone marrow derived mesenchymal stem cells (hBM-MSCs), Conditioned medium (CM), Cell spheroid, Erectile dysfunction (ED)

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1. Introduction

1.1 Research background

1.1.1. Erectile dysfunction

Erectile dysfunction (ED) is a sexual disorder defined as the inability to erect and keep maintenance of erectile state. [1] ED patients are increasing year after year, especially among older adults (ages 40-70), increasing the need for treatment. (Fig.1.A) It is not a disease that is directly threatened by life, but it may cause impacts on psychological and emotional problem.

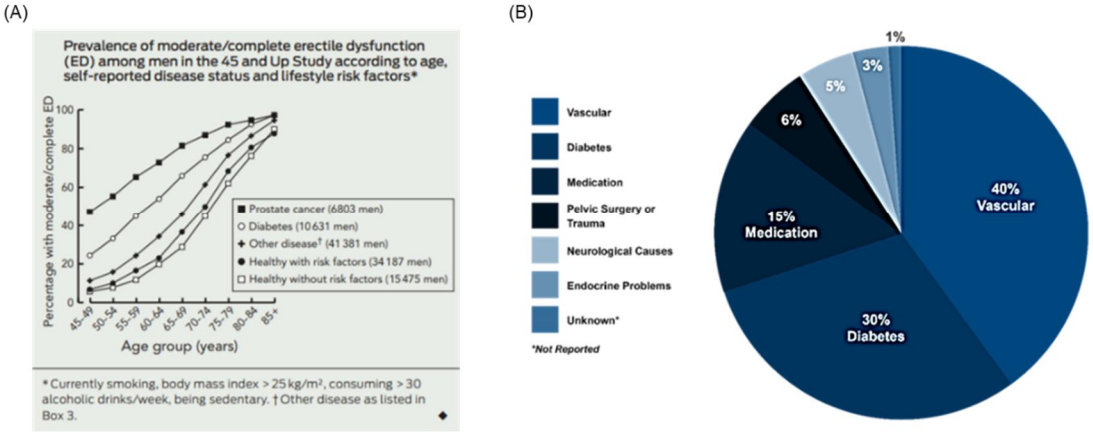

Fig.1. Statistics for several causes of ED

(A) Prevalence of erectile dysfunction among men over 45-years-old (Retrived from [2]) (B) Statistical data on the various physical factors causing ED. (Retrived from [3])

According to statistics (Fig.1.B), problems with blood vessels and diabetes are the biggest part. In addition, pelvic surgery, such as radical prostatectomy (RP), is used to treat prostate cancer. [3] Approximately, 26% to 100% of patients who took RP surgery are estimated to suffer from ED. [4] The prostate and cavernous nerves are located very closely because damage such as cutting and mechanical stretch often damages the endothelium and cavernous nerves of nearby cavernous tissues during RP. These damages make it difficult for neurotransmission by sexual stimulation to the central to peripheral and poor blood supply to cavernous tissues. (Fig.2.) [6]

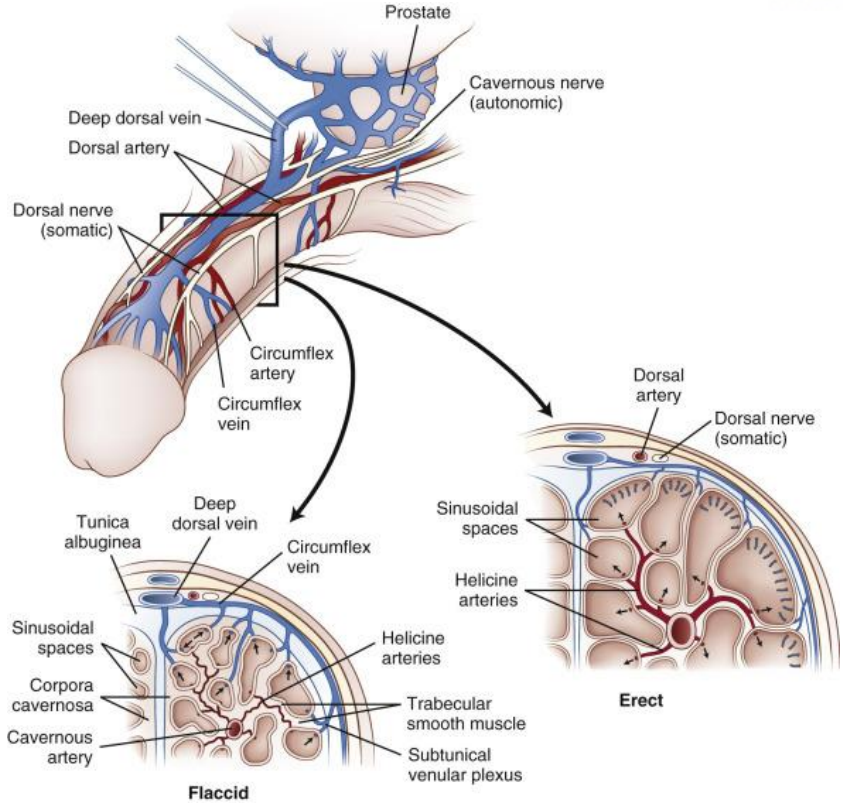

Fig.2. Anatomy of penile tissue during the state in flaccid or erection (Adapted from [5])

As the tissue is damaged, the arterial flow decreases, eventually causing intracorporal hypoxia. Thus, corporal smooth muscle cells are induced to apoptosis and collagen also accumulates. If these smooth muscle cells and collagen are imbalanced, the incoming blood flow leaks through the vein too early to maintain the erection sustained for sexual intercourse. [7]

To sum up, the disease is caused by a combination of factors, not just one, especially endothelial dysfunction and nerve damage. [8,9] Thus, recovery of erectile function requires a combination of therapeutic effects on angiogenesis, neuroprotection and nerve regeneration.

1.1.2. Current therapies for ED

Currently, a variety of treatments are used to recover erectile dysfunction, including medication, stem cell therapy, and gene therapy. (Fig.3.) [10-13]

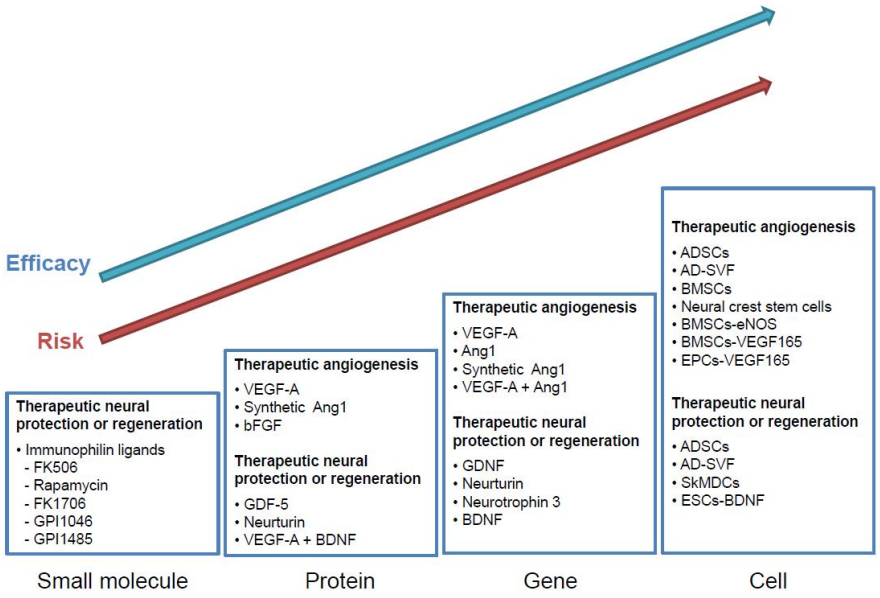

Fig.3. Schematics representing the regenerative therapies for ED treatment

(Adapted from [14])

In related to the pharmacotherapy, it is well-known for their products such as 'Viagra' and 'Levitra'. These drugs act as the phosphodiesterase type-5 (PDE-5) inhibitors. These inhibiting drugs could induce the relaxation of smooth muscle so that makes blood flow into the cavernous tissue. However, the drug treatments show low reactivity due to cavernous smooth muscle and collagen accumulation, making it difficult to expect great effects and complex treatments. [14] Recently, stem cells, for example, mesenchymal stem cell (MSC) are injected into disease areas to overcome the limitations of existing drug treatments, and there are many attempts to perform complex treatments. The use of MSCs has the advantage that complex treatment is possible because it can use cytokines and growth factors related to angiogenesis and neuronal regeneration. [15,16] However, when the cells are injected, a large amount of cells are needed, and even after the injection, the engraftment rate of the cells is low and the tumor may develop into tumors, which is incomplete in terms of stability. In addition, immune responses often occur, requiring the storage of cell stocks, the preparation periods and the high costs that arise from mass culturing for large numbers of cells. [17-19]

1.1.3. Conditioned medium from stem cells

Stem cells have been shown to trigger tissue or organ repair due to their ability to secrete nutrients that have a regenerative effect, rather than the ability to differentiate into constituent cells of damaged tissue. Culture medium in which stem cells were cultured contain lots of secretions called secretomes, microvesicles, or exosomes secreted from stem cells, and the medium containing those secretions is called conditioned medium (CM). [20] This CM contains a variety of growth factors, cytokines, and

extracellular matrix secreted from stem cells. [21]

Recent studies related to the secretomes derived from stem cells have shown that secretomes alone, without the stem cells themselves, could repair the tissue or organ damage rather directly differentiated into the associated tissuees. Thus, in order to make up for the shortcomings of such cell therapy, a CM therapy using only cytokines of cells for its paracrine effect is emerging. CM therapy is a type of protein therapy that can use cell paracrine factors while excluding cell tumorigenesis in conventional cell therapy. CM therapy has the advantage of being ready-to-use because it shows lower immune response and easier to make stock than cell therapy. [22]

Based on these advantages, researchers have recently cultivated stem cells for CM to treat diseases which require angiogenesis and neurogenesis such as wound healing, bone healing, erectile dysfunction, and so on. [23-26] Yutian Dai research group in China harvested a conditioned medium from bone marrow mesenchymal stem cells (BM-MSCs) to confirm its neurotrophic effect. [23] Injecting BM-MSCs or MSC-CMs into the in vivo erectile dysfunction disease model showed more effectiveness for the ED treatment compared to the negative control group. It might be due to the neural regeneration caused by various neurotrophic factors such as BDNF, NGF, and VEGF, which were secreted from the MSCs and included in BM-MSCs-CM. In addition, Hwang's team obtained CM for bone healing using BM-MSCs. The researcher team incubated MSC in a three-dimensional environment using collagen sponge and gave electrical stimulation. [25] As a result, they found more cytokines such as interleukin-8, VEGF, and other factors related to the bone healing. They could identify that more angiogenesis was generated in 3D culture system compared to the conventional method. In addition, Santos's team obtained CM using umbilical cord mesenchymal stem cells. [26] To enhance the paracrine effect of cells, MSCs were cultured in spheroid form so that the angiogenic factors was increased. As such, CM could be obtained from various kinds of cells and is expected to be actively used as a method of regenerative therapy that can treat many kinds of diseases.

1.1.4. 3D spheroid cell culture system

In order to obtain higher protein concentration of angiogenic and neurotrophic-related cytokines from MSCs, various methods such as culture methods, drug treatment and external stimuli have been studied. [24-29] But, among other things, one interesting point is that MSCs cultured in hypoxic condition (<3% O_2) could produce more angiogenic factors compared to the cells in normoxic condition (approximate in 21% O_2). The first method is a gas-controlled incubator that can be cultured by directly supplying gas in hypoxic conditions. In addition, a glove box is also used to make the hypoxic condition of both the subculture and the inside of the incubator where the cell is exposed. In addition, there is a biochemical pseudo-hypoxia induction method that induces a reaction when cells are exposed to a hypoxic environment by treating CoCl2 drug in a culture medium. However, these

methods require the purchase of expensive equipment separately, the treatment of the drug must be removed later, and the cell damage caused by the drug should be considered.

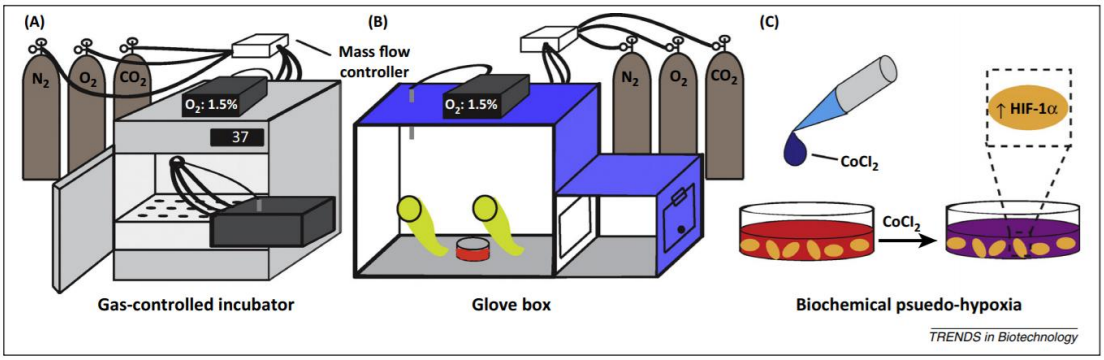

Fig.4. Three methods to induce hypoxic condition for cell culture

(A) The gas-controlled incubator with mass flow controller (B) the glove box (C) biochemical pseudo-hypoxia (Adapted from [30])

Recently, however, researchers have found that mild hypoxic conditions are also established when incubating MSCs in spheroid form. The researchers found that using this method can produce hypoxia more cheaply and easily compared to existing hypoxic condition composition methods. The advantages of the spheroid culture system are that they form hypoxic conditions when the MSCs are cultured in a 3D spheroid form, as well as MSCs in spheroid culture system secrete more growth factors and cytokines than conventional 2D cultures. This is because the cell-to-cell interaction is increased when the cells are cultured in the three-dimensional environment than the conventional monolayer culture method. [29] Thus, the researchers devised a variety of spheroid fabrication methods including suspension cell culture, hanging drop technique, and non-adhesive surface methods. (Fig.5.)

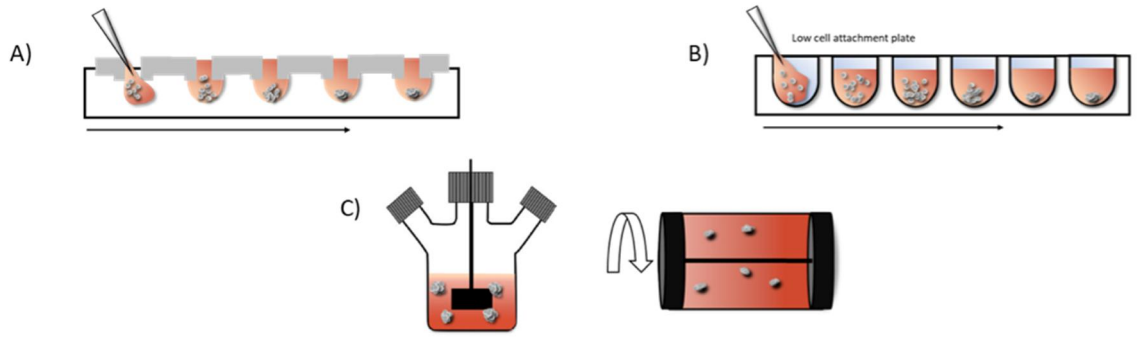

Fig.5. Illustration of methods for spheroid formation

(A) Hanging drop technique to make small droplets of medium containing cells. (B) A method to use the cell culture plated modified with non-adhesive materials. (C) Suspension culture method using spinner flask that stirred the vessels for continuous agitation (Retrieved from google.com)

Suspension culture method is to incubate the cells in suspension, mainly to increase the viscosity or induce spontaneous aggregation through continuous agitation. This method has the advantage of high throughput, but it is difficult to control the size of spheroid to be made, which results in a poor uniformity. The hanging drop technique is often used because it requires no tools. It is a method of inducing cell spheroid formation by gravity force by hanging droplets containing cells on the underside of the lid. Although the size control is relatively easy than the previous method, it is not suitable for mass production due to the difficulty in mass production. Lastly, non-adherent surfaces are used to create spheroids. It is based on the simple concept that cells could make aggregated form by themselves when they are cultured in the plate modified with a material without cell binding site or cultured in a mold made of agarose. This method can also produce spheroids of uniform size with high-throughout. [31]

1.2 Research Objectives

As mentioned above, a research method of harvesting high concentration of CM by maximizing the paracrine effect of stem cells should be established. The main goal of the study is to optimize the method of increasing the concentration of protein secreted from bone marrow and to identify the effect of concentration on the actual erectile dysfunction model. In particular, we were interested in quantitatively comparing and evaluating the rate of increase of concentration when cells were cultured using the 3D spheroid culture system compared to the conventional culture method. In addition, the concentration rate was also evaluated according to the centrifugation time using the filter unit to achieve a therapeutic effect even with a small volume. This study aims at preliminary studies to obtain CM with

increased angiogenic and neurotrophic effects. The results should be validated in erectile dysfunction in vivo models due to cavernous nerve injury, which may provide a safer and more efficient treatment method for not only cavernous tissue repair but also tissue regeneration.

2. Materials and Methods

2.1. Cell cultures

Human bone marrow mesenchymal stem cells (hBMMSC, Lonza, Basel, Switzerland) were cultured in low glucose Dulbecco's Modified Eagle's Medium (DMEM, Welgene, Korea) supplemented with 10% fetal bovine serum (FBS, Capricorn, Ebsdorfergrund, Germany), and 1% penicillin/streptomycin (P/S, Capricorn), and grown until 70% confluency. Green fluorescent protein tagged human umbilical vein endothelial cells (GFP-HUVEC, Angio-proteomie, USA) were cultured in Endothelial Growth Medium (EGM-2, Lonza, Basel, Switzerland).

2.2. Preparation of microwell for spheroid culture

The microwell was fabricated by using 12-series micromold (The 3D Petri Dish®; Merck KGaA, Darmstadt, Germany). Briefly, 1 g of agarose powder (Invitrogen, Carlsbad, California, USA) was added into 50 mL of saline solution. Then, the mixture was boiled and injected into the micromold. After cooling for gelation, the microwell was taken off from the mold and put in the 12 well plates (Corning, NY, USA).

2.3 Preparation of BM-MSCs-conditioned medium

For 2D culture, in 80% confluent, MSCs in 6-well plates (Corning, NY, USA) were fed with 2 mL of serum-free DMEM per well for 48 hours. For 3D culture, MSCs were put in the agarose micromold. Cell densities for developing spheroids in various diameter (150, 300, and 450 μm); 6.83×10^6 cells/well, 2.73×10^7 cells/well, 9.33×10^7 cells/well for each spheroid. After formation of spheroids, the spheroids were fed with 2 mL of serum-free DMEM per well for 48 hours. After 48 h, the conditioned medium was collected and concentrated by using 3-kDa cut-off centrifugal filter units (Merck, Darmstadt, Germany) following the manufacturer's instructions. hBMMSC-CM was stored at -80 . Protein concentrations were determined using the bradford assay reagent (Sigma Aldrich Co., St. Louis, PA, USA).

2.4. Cell viability test

For viability assay, a live/dead Assay kit (ThermoFisher Scientific, Waltham, MA, USA) was used at 48 hours before harvesting CM as described by the manufacturer. Cells were incubated with 0.5 μL

/ml calcein-AM and 2 μL/ml of ethidium homodimer-1 for 1 hour at RT, and then viability was assessed by observation using a fluorescence microscope (Leica DM2500, Leica Microsystems AG, Germany).

2.5. Enzyme-linked immunosorbent assay (ELISA)

The concentration of angiogenin (ANG), vascular endothelial growth factor (VEGF), and brain derived neurotrophic factor (BDNF) in hBMMSC-CM were assessed using a commercial ELISA kit (R&D system, Minneapolis, MN, USA). The concentration of glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) in CM were analyzed by using a commercial ELISA kit (Raybiotech, Norcross, GA, USA), following the protocols provided by the manufacturer.

2.6. EC tube formation assay

Firstly, 289 μL of growth factor-reduced Matrigel (GFR-Matrigel) (10 mg/ml, Corning, NY, USA) was coated on a 24-well plate. Next, $1.5x10^5$ GFP-HUVECs were seeded in 300 μL of diluted hBMMSC-CM or not diluted CM. CM was diluted with serum-free culture medium. After 4 hours of incubation at 37˚C, the wells in the plate were photographed by a fluorescence microscope. The average of the number of nodes, meshes, and the number of total tube length of four images was photographed as the value for each treatment. The number of nodes and meshes was analyzed using Image J software manually.

2.7. Animal and study design for in vivo study

All aspects of animal study were accepted by the Institutional Animal Care and Use Committee of Asan Medical Center (2018-13-155). Fifty male Sprague-Dawley rats of 8-week-old were purchased from Orient Bio Inc. (Gyeonggi, Korea). The 9-week-old-rats after acclimatizing for a week were divided into 5 groups and each group got 10 rats in random : sham group, control group, low-dose injection (3.2 μg/uL, ‘Low’), moderate-dose injection (21 μg/μL, ‘Moderate’), and high-dose injection (42 μg/μL, ‘High’). Then, laparotomy was conducted for the sham group. In the other 4 groups, bilateral CN injury was performed. Phosphate-buffered saline (PBS), and each group of CM were injected intracavernously into the rats, respectively. After 4 weeks, erectile function evaluation was measured. The tissues were prepared for the histological experiments after sacrificing rats.

2.8. CN injury and CM injection

The procedures were performed as described previously. [32] Briefly, animals were anesthetized with 0.2 ml of tiletamine (Zoletil) intramuscularly. After detecting bilateral CNs on the lateral side of the prostate, CNs were separated from the prostate. By giving compression with two mosquito clamps the CNs were subsequently injured for 2 minutes. CM or PBS in 50-μL volume were intracavernously injected into the corpus cavernosum by using a 30-G needle. After 1 minute from injection, the compression was released.

2.9. Erectile function evaluation and tissue preparation

The erectile function was evaluated as described previously. [32] After 4 weeks of injection, 200 μL of tiletamine was used to anesthetize all rats intramuscularly. The major pelvic ganglion (MPG) set forth on posterolateral to the prostate. A continuous pressure transducer was connected to the cannula in real time to measure mean arterial pressure (MAP). For the measurement of intracavenous pressure (ICP), a heparinized 23-G needle inserted to the corpus cavernosum. The MPG nerve was stimulated at a frequency 10 Hz. Electrical stimulation with 5 V for 60 seconds was applied to the nerve with a period of at least 10 minutes between subsequent stimulations. ICP in the maximum and total ICP were divided by the MAP and expressed as ICPmax/MAP and ICPtotal/MAP respectively. The penil tissues of each rat were harvested for histomorphometric analysis after evaluation of erectile function.

2.10. Immunohistochemistry staining of Penis Tissue

As described in detail previously, [32] penile tissue sections in 5-mm of thickness were prepared for immunohistochemistry after erectile function evaluation. Firstly, tissue sections placed on micro-slides were deparaffinized with xylene. Then, the sections were hydrated in alcohol solutions, and immersed in 3% H2O2 to put out endogenous peroxidase activity.

Primary antibody, anti-von Willebrand factor (vWF) (1:400; Abcam), was incubated with the tissue sections for 2 hours at RT. After washing, it was incubated with a biotin-free polymeric horseradish peroxidase-linker antibody conjugate system (Lab Vision) for 30 minutes. After washing, chromogen development was proceeded for 10 minutes. Prior to examination, Mayer's hematoxylin was used to counterstain and then the slide was mounted using Immu-Mount (Thermo Shandon). To stain fibrers with red, the sections were incubated with Biebrich scarlet-acid fuchsin solution for 5 minutes. Then, samples were stained with aniline blue for 3 minutes. Next, the samples were incubated with 1% glacial acetic acid to get accurate and translucent colors. In the next step, the slides were washed in distilled water, dehydrated and cleared. Samples were mounted by using dibutyl phthalate xylene (Sigma-Aldrich). Four regions of the sections were randomly selected per each rat and photographed to analyze images. To quantify the contents of ECs and smooth muscle in cavernousum, Olympus U-LH100L-3 camera (original magnification, ×20) and Olympus Ix 71 software were used for getting

images of the sections. ImagePro-Plus 5.1 software (Media Cybernetics) was used to analyze all images.

2.11. Immunofluorescence Staining of Penis Tissue

As described in detail previously, [32] samples were fixed in 4% paraformaldehyde and blocked with normal goat serum. For smooth muscle and endothelial contents staining, anti-α smooth muscle actin (αSMA) (1:400; Sigma-Aldrich), anti-endothelial nitric-oxide synthase (eNOS) (1:100; Becton), anti-vWF (1:400; Abcam) were added. For neuronal staining, anti-neuronal nitric-oxide synthase (nNOS) (1: 100; Novus Biologicals) was used. The blocked samples were incubated with primary antibodies overnight in a humidified chamber at 4°C. Nuclear was stained using 4,6-diamidino-2-phenylindole (Vector Laboratories). Goat anti-mouse and goat anti-rabbit (1:400; Thermo Fisher Scientific Inc.), were used as secondary antibodies and incubated for 1 hour at RT. To analyze the images, four fields of tissue sections were chosen in random. The images were photographed using fluorescence microscopy (Carl Zeiss). For αSMA staining, the section was examined at 200-magnification and for nNOS and eNOS, the original magnification was 400. All images were analyzed using Adobe Photoshop CS2 (Adobe Systems Inc).

2.12. Statistical Analysis

SPSS statics (version 21) or GraphPad Prism software (version 5.01) and was used to perform statistical analysis. All data were expressed as means ± standard error of the mean (S.E.M). Statistical comparisons between experimental groups were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey's honest significant difference test for post hoc comparisons. A p-value<0.05 was considered as statistically significant.

3. Results

3.1. Production of BMMSC spheroid and confirmation of size and viability

We confirmed that the spheroids of the desired diameter were formed according to the concentration of cells in the agarose micromold, and the survival rate was confirmed by fluorescence staining. Fig.6.A shows the survival rate of cells in spheroids using the live/dead assay. The overall survival rate was high. But the larger the diameter, the more dead cells were observed. In addition, it was confirmed that the size of the spheroid also increases with increasing cell concentration.

3.2 Comparison of the concentration of secreted proteins in CM from 2D monolayer/3D spheroids

The concentration of protein secreted from MSCs cultured in 2D and the amount of those in 3D were compared by bradford assay (Fig.6.B). The concentration of protein secreted from cells cultured in 2D or 3D was normalized to initial seeding cell density. The amount of secreted protein per single cell increased by 278 times for the diameter of 150 μm spheroid, 75 times for 300 μm spheroid, and 19 times for 450 μm spheroid. Therefore, we decided to obtain CM by making a spheroid with a diameter of 150 μm with the highest amount of protein secretion per cell.

3.3 Protein Concentration with Centrifugation Time

Experiments were conducted to determine how the total protein concentration of CM was concentrated along the centrifugation time. The total protein amount was determined by bradford assay at 30, 50, 70, 90 and 110 minutes for centrifugation (Fig.7.B). As a result, compared with the total protein concentration of the CM centrifuged for 30 minutes, it was confirmed that about 19 times increased for 110 minutes. Afterwards, to determine the types of various proteins in CM and to investigate the concentration of angiogenic and neurotrophic growth factors, the membranes with the protein antibody array were incubated with CM for 30 or 110 minutes. (Fig.7.A) Human XL cytokine arrays incubated with CM revealed several chemokines, growth factors and other trophic factors. Most cytokines responded positively to blotting. Among angiogenic factors, MMP-9, ANGPT1, ANG, and VEGF showed positive blotting. The concentrations of angiogenic factors such as ANG, ANGPT-1 and VEGF were found to be highly concentrated with increasing centrifugation time. (Fig.7.C) In the case of neurotrophic factors, growth factors such as BDNF, bFGF, LIF, and TGF-alpha responded. The neurotropic factor such as BDNF and bFGF also showed a similar tendency to

angiogenic factor but had lower concentrated ratio. From this, it can be seen that even with increasing centrifugation time, not all factors are concentrated at the same rate. The 110 minute-time is longer than the recommended time of 15-60 minutes, which is the recommended protocol of the filter centrifugal tube. Concerned about low sample recovery due to adsorptive losses, it was decided that CM concentrated for 90 minutes would be used in subsequent experiments. Then, the concentration of growth factors in the CM was measured by ELISA. (Fig.7.D,E) As a result of measuring the concentration of key angiogenic factor in the concentrated CM, ANG showed 1503.4 ± 191.5 pg/mL and VEGF contained 3811 ± 352 pg/mL. In addition, key neurotrophic factor concentrations were 12.6 ± 3.3 pg/mL, 4.3 ± 0.036 pg/mL, and 74.7 ± 1.8 pg/mL for GDNF, NGF and BDNF, respectively.

3.4. Idendification of angiogenic effect of CM through EC tube formation assay

In order to confirm the angiogenic effect of CM, the tube formation assay using HUVECs was confirmed by Matrigel tube formation assay. (Fig.8.) The tube formation assay showed that ECs treated with CM 100%, CM 75%, CM 50%, and CM 25% induced more tube formation than those treated with DMEM (negative control) only. Based on the fluorescence images, the number of nodes and meshes and the total length of the formed tube were quantitatively analyzed using Image J software on angiogenesis criteria. (Fig.8.B-D) The CM 100% group showed an increase of total tube length about 14 times compared to the DMEM group. Furthermore, the number of meshes and nodes increased 24 times and 15 times, respectively. In the case of mesh and length, 25% of the CM was treated, which is very significant. Taken together, we found that the angiogenic effect increased with CM concentration in all results. These results show that hBM-MSC-CM has angiogenic effect, which is concentration-dependent.

3.5. Effects of hBM-MSC-CM on in-vivo Erectile Function

During MPG stimulation, ICPmax/MAP confirmed that the sham group was significantly higher than the control group (0.55±0.28 vs. 0.25±0.18). Intracavernous injection of CM showed no significant difference in erectile function compared to control group, but it was slightly improved in each CM group as there was no significant difference between low, moderate, and high group compared to sham group. (Fig.9.)

3.6. Estimation of therapeutic effect on cavernous fibrosis

The contents of smooth muscle cell and collagen was quantified by Masson's trichrome (MT) staining and immunofluorescence staining. (Fig.10.A, B) As a result, the smooth muscle/collagen ratio was significantly higher in the sham group compared to the control group and considerably increased in the CM treated group. Additionally, as the concentration of CM was increased, all ratios were significantly increased. (Fig.10.C) In addition, αSMA expression revealed the increasement of the smooth muscle contents when treated with CM. The low and moderate groups showed marginally increased, but very significant values were measured in the high group. (Fig.10.D) Based on those data together, when the CM was injected, smooth muscle content increased compared to the control, and the higher the concentration was, the more effective it was.

3.7. Endothelial cell content

Immunofluorescence and immunohistochemical staining revealed that eNOS-positive stained area per carvernosum of the sham group was higher than that of the control group, indicating high endothelial cell content. (Fig.11.A, B) Referring to Fig.11.C and D, the CM-injected groups showed a significant increase in the endothelial cell content compared to the control group. In addition, in the case of endothelial cell content, even low-dose injection was effective in increasing EC contents and showed a significant difference according to the concentration in each concentration group.

3.8. Neurotrophic effect of hBM-MSC-CM for CNs

Intracavernous injection of BM-MSC-CM promoted nerve regeneration in the penile dorsal nerve, marginally in the low- and moderate-dose groups and significantly in the high-dose group. (Fig.12.) There was no significant difference according to the concentration. However, nNOS positive area was increased along with increase of the concentration of CM, the result also showed that nerve regeneration was dependent on the concentration.

4. Discussion

Recently, many regenerative medicine studies have been conducted in addition to ED treatment using stem cells isolated from numerous sources such as adipose tissue, bone marrow and umbilical cord blood. [12,23,33] In the early treatment of ED, MSCs received a lot of attention due to their homing ability into damaged tissue and the ability to differentiate into the cells in the injured tissues. [34] In particular, BM-MSCs are being used for ED treatment because of the increasing evidence of paracrine effects that promote tissue repair. [23,35-37] BM-MSC has been shown to promote neuronal regeneration by secreting neurotrophic and angiogenic growth factors such as BDNF, NGF, VEGF, etc. as well as having trophic effects related to anti-apoptosis and immunomodulation. [23] However, few studies have shown long-term survival and high engraftment when stem cells are implanted directly, and tens of millions of cells are needed for transplantation. [17-19] In addition, controversy such as safety and tumorigenesis still remains associated with MSC. Therefore, many researchers are trying to use the various secretomes produced by MSCs rather than directly transplanting cells. [38-40] CM is a representative way to overcome this disadvantage. However, because the CM obtained from MSCs simply grown in a 2D culture dish has low concentrations for therapeutic effects, the researchers are interested in ways to obtain high concentrations of CM. [23,25,29] In this study, 3D spheroid culture system was used to provide mild hypoxic condition to MSC. Agarose mold without cell binding site was fabricated to make cell spheroid. It is more inexpensive than the hypoxic chamber used for the formation of hypoxic conditions, and has the advantage that it is possible to produce spheroids of a more uniform size than the spheroid making method such as the hanging-drop technique and spinner flask, and is easy to make. [31,41]

It was judged that the larger the diameter, the more dead cells were seen as the diameter was increased although the viability of the spheroid produced could not be calculated with the correct value. In addition, the total protein concentration was high when the diameter was 450 μm, but when normalized to the initial seeding density, the protein production produced per cell by the spheroid with the diameter of 150 μm was the highest. Therefore, it was determined that spheroids of 150 μm produced the highest concentration with the best efficiency, and were adopted as a condition for later production of spheroids. One interesting point is that the amount of protein produced per cell in a relatively large spheroid of 450 μm did not differ significantly from the amount of protein produced in a typical 2D cell cultivation system. It might be due to the fact that too large spheroid size leads to more dead cells and more severe hypoxic condition, resulting in reduced protein production. [42-44] Next, the concentration ratio of the total protein concentration and the key growth factors was compared with the centrifugation time using the centrifugal filter. Concentration of each protein was expected to be similar when the whole protein was concentrated, but protein concentrations such as LIF and TGF-α did not increase significantly with increasing centrifugation time. When referring to [45], since the structure of the protein is not spherical

but various shapes such as linear, random, it is estimated that it could pass through the filter membrane even though it has a size of more than 3 kDa when concentrated.

Recent studies revealed that intracavernous injection of BM-MSC-CM induced erectile function restoration due to the neuroregenerative effect of MSC. [23,35-37] In this study, we confirmed that MSC has a very broad spectrum of proteins such as growth factors, chemokines, anti-inflammatory factors, and immune modulation factors using array membranes coated with various antibodies. Among the proteins known as ANG, VEGF, GDNF, BDNF, and NGF, which are known as key growth factors, the values obtained by ELISA measurement of the concentrations were sufficient to be detectable as mentioned in [23], so it was determined that there were angiogenic and neurotrophic factors in CM that could affect the therapeutic effect on ED.

Angiogenic effect was confirmed by tube formation assay using GFR-Matrigel. EC seeded on Matrigel had active tube formation from 4 hours. Thus, we examined node, mesh, and tube length, which are often referred to as criteria for angiogenesis at 4 hours. [46] In the case of node, CM 25% and DMEM group did not show a big difference, but in the case of the number of mesh tube lengths, there was a significant difference between those two groups. Particularly, all three values increased significantly according to the increase in the concentration of CM in the case of the number of meshes and the tube length.

These in vitro results were similarly confirmed in vivo. We divided the five groups according to the CM concentration and confirmed that the erectile function was restored by intracavernous injection in the CN injury rat model. Moreover, it was identified that the CM treatment had a recovery effect due to the anti-apoptosis effect on smooth muscle cells in relation to the increase of ratio of smooth muscle to collagen. And it has similar result in increased endothelial cell contents related to the angiogenic effect. Additionally, nNOS-positive nerve fiber was also significantly increased, and CM promoted neuronal regeneration as the concentration of CM with angiogenic and neurotrophic effects increased.

In summary, we established the method for harvesting CM with high concentrations of protein and identified that it contained high growth factors and cytokines related to angiogenic and neurotrophic factors. In addition, the therapeutic effect of CM treatment to erectile dysfunction animal model was confirmed. Furthermore, CM is expected to be effective in the treatment of disease models that require angiogenesis and neurotrophic effects, such as neurodegenerative disease or ischemia disease.

Figures

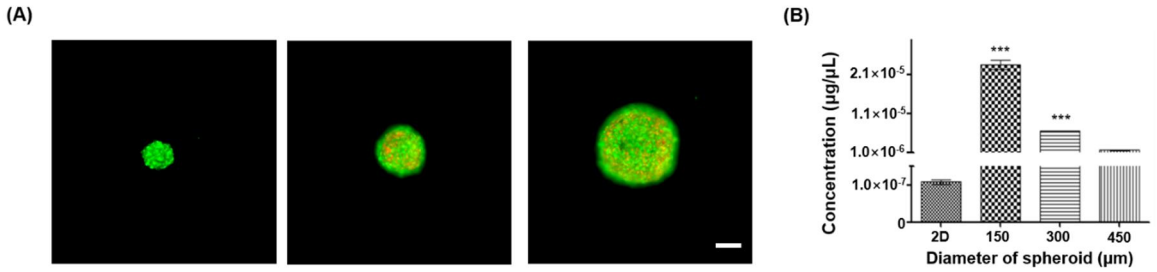

Fig.6. Effect of spheroid diameter on the cell viability and total protein concentration of CM

(A) Fluorescent images resulting from live/dead assay. Green and red color indicates live and dead cells, respectively. Scale bars; 100 μm. (B) Total protein concentration of CM using bradford assay. Statistical analysis was performed by a one-way ANOVA, followed by Tukey's comparison tests. Data are shown as mean±S.E.M (n=3). *** indicates p<0.001 compared to 2D group.

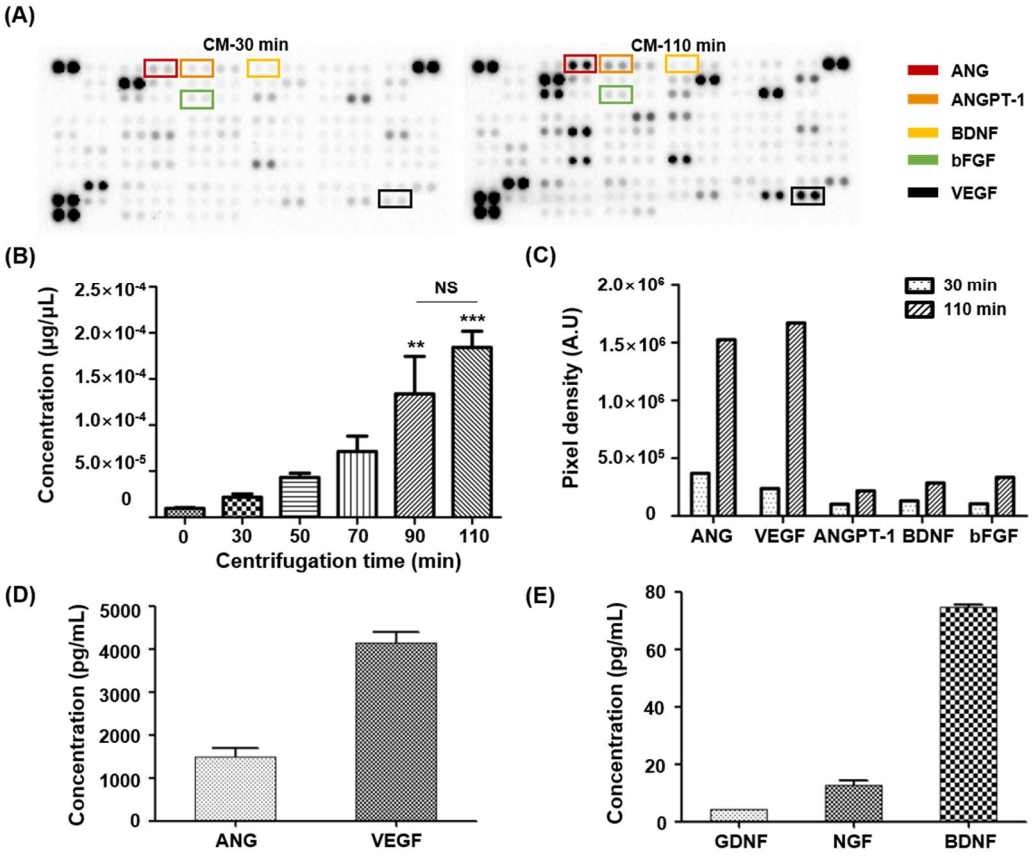

Fig.7. Effects of centrifugation time on growth factors in CM

(A) Human Cytokine Antibody array incubated with CM-30 min (left panel) and CM-110 min (right panel). Boxes indicate growth factors which are related to the angiogenesis and neurogenesis. (B) Total protein concentration of CM along the increased centrifugation time. NS indicates no significance, **, p<0.01, ***, p<0.001 compared to control group. (n=3 samples

per group) (C) Concentrated angiogenic factors and neurotrophic factor. Concentration of key angiogenic factors (D) and neurotrophic factors (E) by using ELISA. Data are expressed as mean ± S.E.M (n =5 samples per group).

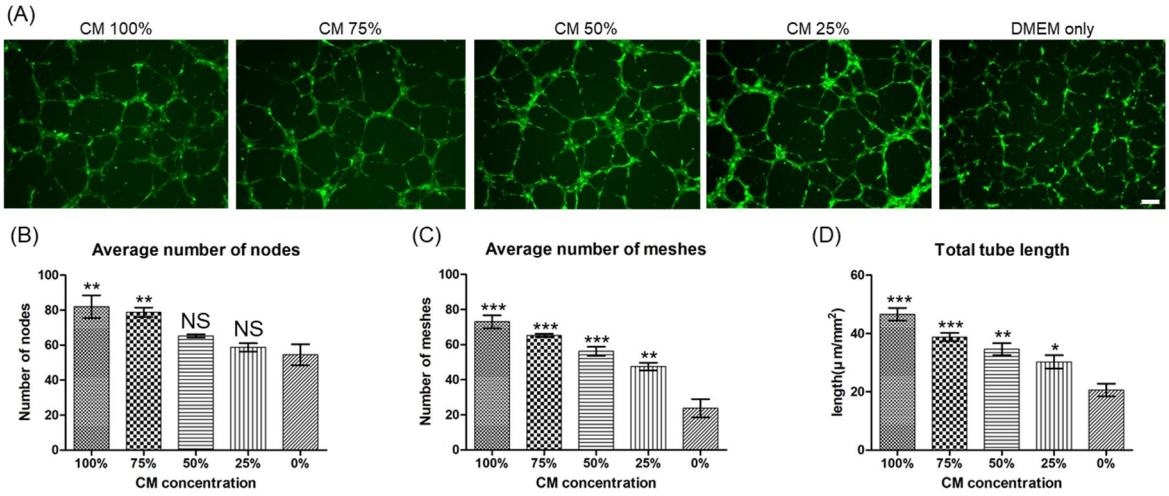

Fig.8. Identification of angiogenic effect of CM by EC tube formation assay

(A) Representative fluorescence images of HUVECS on Matrigel incubated with each group of CM. (B)-(D) Quantitative analysis based on fluorescent images for average number of nodes, meshes, and length, respectively. Statistical analysis was performed by one□way ANOVA, followed by Tukey's comparison tests. NS indicates no significance, *,p<0.05, **, p<0.01, ***, p<0.001 compared with DMEM only group (0%). Data are expressed as mean ± S.E.M (n=4 samples per group).

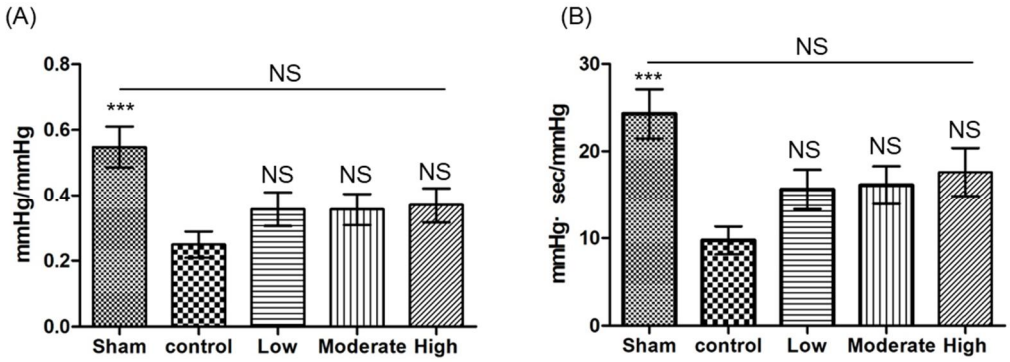

Fig.9. Erectile function evaluation with ICP/MAP ratios

(A) Ratios of ICPmax/MAP (B) Ratios of ICPtotal/MAP. NS indicates no significance (p> 0.05 between the control and CM injection groups); ***, p< 0.001 compared with the control group.

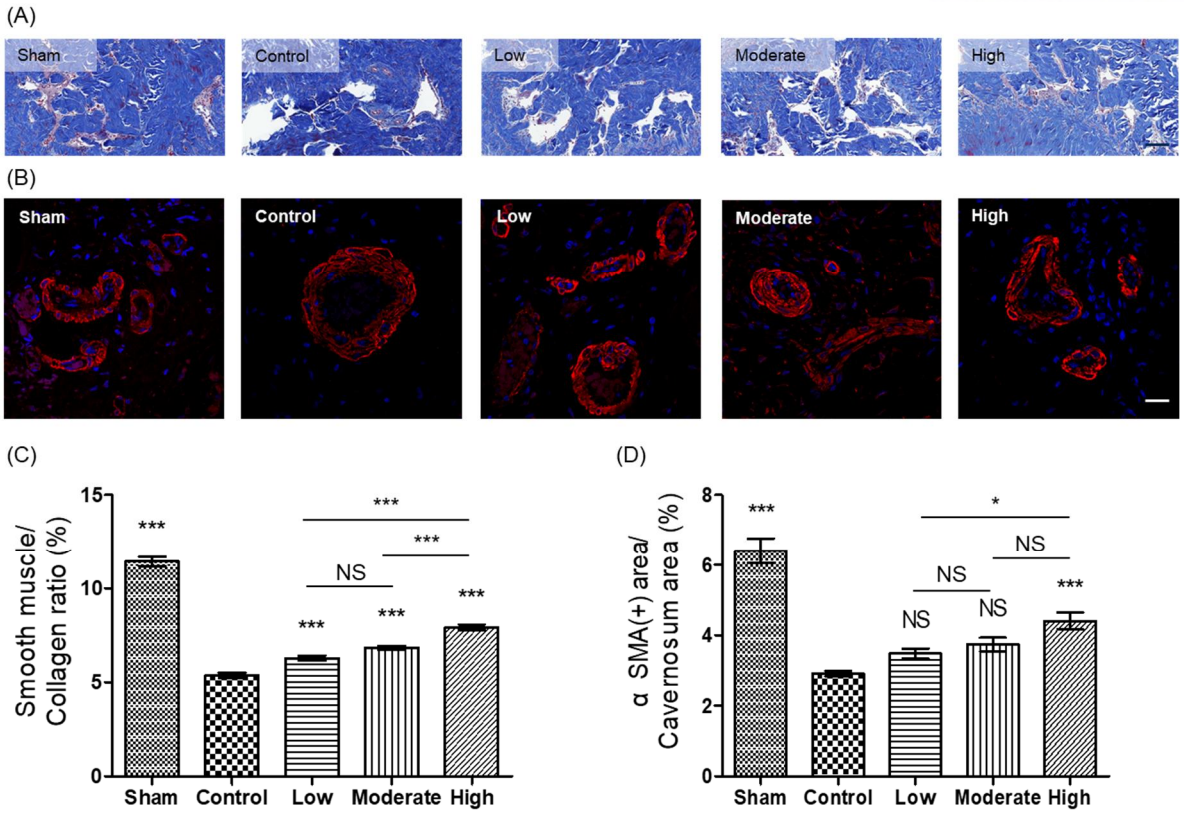

Fig.10. The ratio of smooth muscle to collagen

(A) Representative images of Masson's trichrome stained corpus cavernosum tissue. Original magnification, ×20 (B) Representative images of corpus cavernosum tissue expressing αSMA. Original magnification, ×200 (C) Quantitative image analysis of MT stained images (D) Quantitative graph showing the ratio of positive αSMA area to the total cavernosum area. NS indicates no significance; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with the control group;

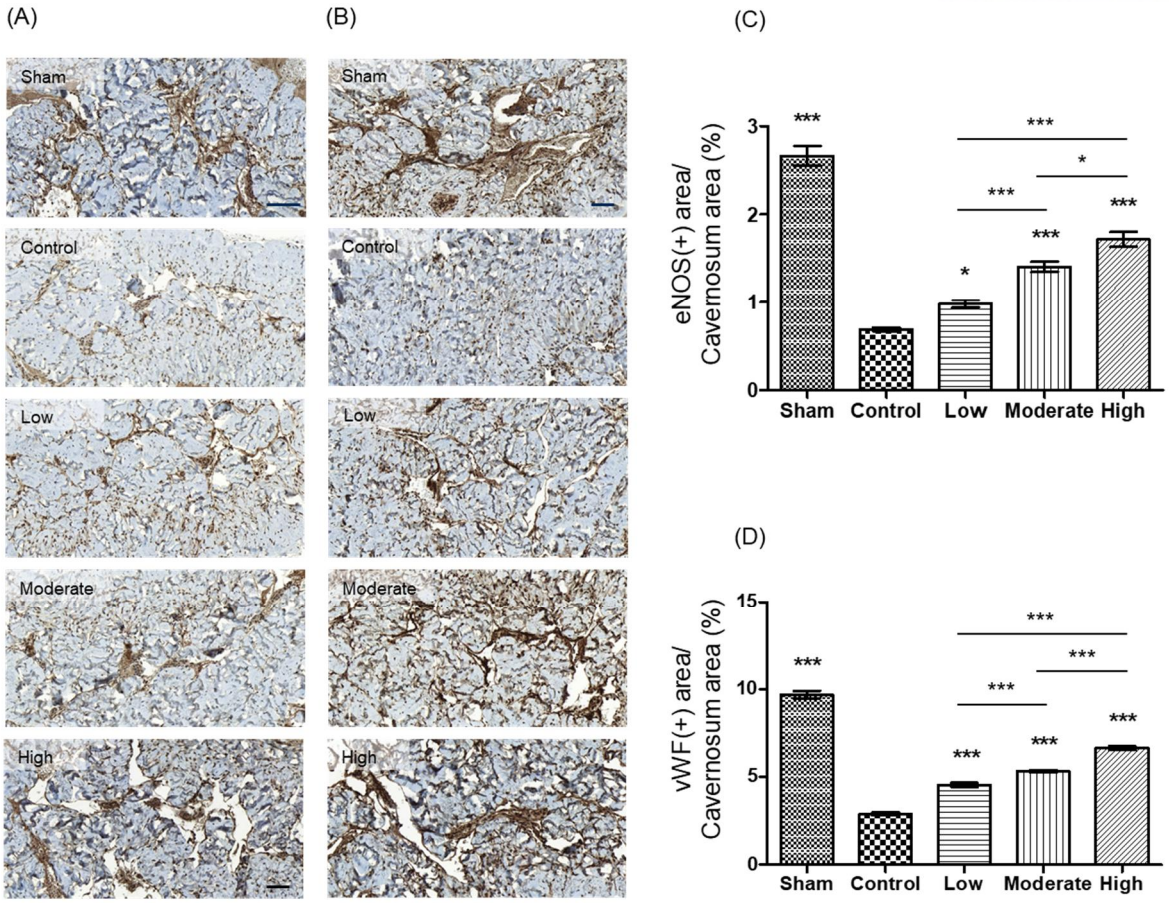

Fig.11. Endothelial cell contents in corpus cavernosum

(A) Representative images of eNOS stained corpus cavernosum tissue. (B) Representative images of vWF stained corpus cavernosum tissue. (C) The graph represents eNOS positive stained area/cavernosum area. (D) The graph represents eNOS positive stained area/cavernosum area. Cavernosum area was chosen which was within the tunica albuginea. Original magnification, ×20. * indicates $p<0.05$; ***, $p<0.001$ compared with the control group.

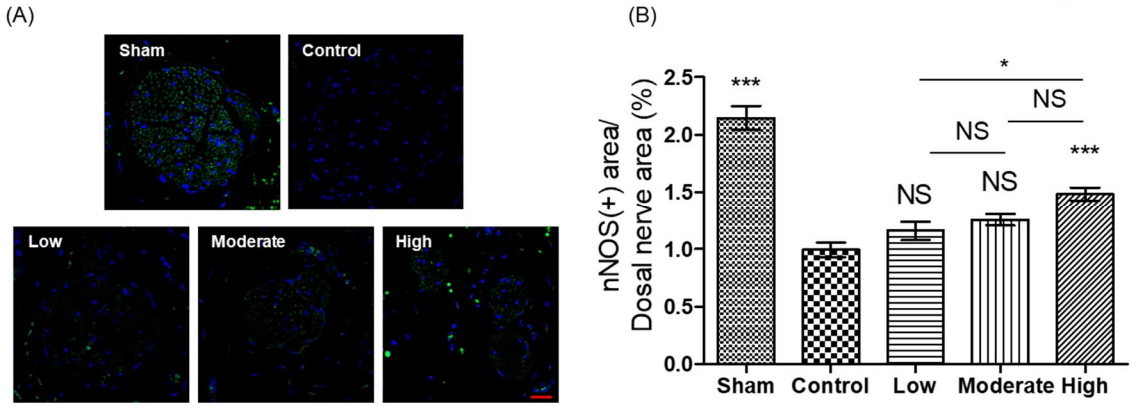

Fig.12. Identification of neurotrophic effect with immunofluorescence staining of nNOS

(A) Representative images of nNOS expression for each group. Original magnification is ×400. (B) Quantitative result of positively stained nNOS within the dorsal nerve area. NS indicates $p>0.05$; *, $p<0.05$; ***, $p<0.001$ compared with the control group and each CM treated group.

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