Abstract

Fabrication of micro-cryogels for therapeutic angiogenesis

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Peripheral artery disease is a most common symptom in diabetic patients. Recently, enhancing neo-vascular angiogenesis has been emerged as a promising therapeutic solution.

Herein, we tried novel genetically edited stem cell delivery carrier as vascular endothelial growth factor (VEGF) transfected human tonsil derived mesenchymal stemcells (hTMSCs), which have been known as multipotent cells, within micro cryogel. Micro-cryogel was constructed with hyaluronic acid (HA) added poly ethylene glycol (PEG) which is reported as biocompatible. We dropped solution of hydrogel on initiated chemical vapor deposition (iCVD) processed hydrophobic surface. Hydrogel, then forms spherical structure, because of its own hydrophilicity. Chemically cross-linked gel were frozen and lyophilized. Manufactured cryogels were harvested and VEGF transfected cells were cultured within them. Transfection efficiency was controlled over 40%. Furthermore, size of cryogels could be

controllable. VEGF secretion was confirmed with fluorescence measurement and quantified with VEGF ELISA assay. Culturing hTMSCS within gels not only prolonged VEGF releasing duration for several days but also maintained secretion level stably. Also, hTMSCs with PEG-HA cryogels demonstrated therapeutic effect on hindlimb ischemia model *in vivo*. Therefore, we suggest that our newly developed micro cryogel system could be a promising new remedy for not only diabetic but also all others who suffered from deteriorated blood perfusion.

Keywords : Human tonsil derived mesenchymal stem cells, Stem cell therapy, Drug delivery, Hyaluronic acid, PEGDA, Cryogel, Neovascularization

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1. Introduction

A variety of therapeutic cell therapies with the aim of enhancing neovascularization has been progressed over the past few decades. This manner was then used as treatment for most of blood perfusion disease such as myocardial infarction, diabetes or peripheral arterial disease [1-3]. However, many researchers have been faced one bottleneck that environment of ischemic disease area is often inflammatory and hypoxic [4,5]. This condition will led cells to apoptosis, thereby limited cell injection efficacy was observed [4]. Thus, in order to overcome these drawbacks, using genetically modified cells has the reasonable possibility to increase cell delivery efficiency by upregulating angiogenic or anti-apoptotic factors within cells. For instance, genetic modified mesenchymal stem cells (MSCs) with the anti-apoptotic factor (Bcl-2) improved efficacy of cell therapy in *in vivo* model [6]. These methods possibly promote the medicinal potential and conquer the inferior cell viability at the transplanted region for facilitating angiogenesis. This indicated that collateral blood vessel regeneration and capillary reconstruction could be induced through angiogenic signal pathway mechanisms mediated through vascular endothelial growth factors (VEGF) and stem cells [7]. However, viral vectors still have many problems like host infection. So, other approaches, such as polymeric transfection or electroporation is now arising.

Among the other approaches for improving efficacy of cell therapy, cell encapsulation technology was risen as a promising approach to prevent an apoptosis of injected cells at ischemic site. Also it was considered that biocompatible materials are necessary to improve the therapeutic efficacy of cell-based cure *in vivo*. Recent studies also showed that various materials, including antibody microgels, poly (D,L-lactic-co-glycolic acid) (PLGA) and

alginate hydrogel encapsulated cell carriers significantly enhanced cell viability and stability after transplantation [10, 11]. Among them, PEGDA which is biocompatible and biodegradable material was widely used as hydrogel carrier for the drugs, proteins, and cells. It has been demonstrated that these various feaatures have a desirable ability which could protect therapeutically delivered cells from a host immune system [10].

In accordance with previous study, hyaluronic acid (HA) can facilitate cell attachment and migration into the scaffold due to its high water retention and intrinsic swelling property [12]. HA has been widely utilized as a beloved hydrogel material for the transplantation for various cells, including chondrocytes, osteoblasts, endothelial cells, and mesenchymal stem cells [13].

In this study, we developed PEG-HA micro cryogel system which encaspsulated VEGF transfected hTMSCs to induce enhanced neovascularization for hindlimb ischemia

2. Materials & Methods

2.1 Fabrication of hydrophobic substrate via iCVD.

To introduce hydrophobicity onto bare glass substrate, 1H, 1H, 2H, 2H-perfluorodecyl methacrylate (PFDMA) was deposited via initiated chemical vapor deposition (iCVD) process. Both the monomer, 1H,1H,2H,2H-perfluorodecyl methacrylate (PFDMA)(Sigma Aldrich) and, and the initiator, tert-butyl peroxide (TBPO) (Sigma Aldrich), were used without any further purification from purchased state. Both materials were loaded separately into source cylinders, which were added to the iCVD reactor (Daeki Hi-Tech., Korea).[14]

2.2. Characterization of hydrophobic substrate.

X-ray photoelectron spectroscopy (XPS) survey scans were performed with Sigma Probe Multipurpose XPS (Thermo VG Scientific, USA) with a monochromatized Kα source. Water contact angles of bare glass and PFDMA substrate were measured using a Contact Angle Analyzer (Pheonix 150, SEO, Inc.). The volume of water droplet introduced to each sample was 6 μL[14].

2.3 Fabrication of PEGDA cryogels

PEGDA based ECM-mimicking cryogels were made by mixing PEGDA 20% (w/v) dissolved in phosphate buffered saline (PBS) with MeHA 20% (w/v) also in PBS as 1:1 volume ratio. Thus, final concentration of both PEGDA and MeHA became 10%. To this mixed

solution at 4℃, ammonium per-sulfate 0.5% (w/v) (APS; Sigma-Aldrich) solution and either 0.05% (w/v) or 0.25% (w/v) of N,N,N',N' -tetramethylethylenediamine (TEMED; Sigma-Aldrich) solution was added to the final desired concentrations of 4% (v/v)[15]. Then, polymer solution was dropped on iCVD coated glass plate and placed in the freezer. During polymerization, this mixture was stored in -20℃ for 20h. Interconnected and macro pores were created by thawing ice crystals within scaffold. Two different ratio of TEMED were used to measure the effect of concentration of TEMED on the degree of ice crystals formation within scaffold structure. Prior to cell seeding, scaffolds were washed several times with sterile PBS to remove unreacted residues and then, sterilized with UV for 20 min. Cells were seeded on different types of scaffolds: PEGDA 10% (w/v) (PEG) , PEGDA + MeHA 5% (PEG-HA5), and PEGDA + MeHA 10% (PEG-HA 10) at a concentration of 1×10^4 cells / μl in a drop wise manner[16].

2.4 Scanning electron microscopy

Lyophilized scaffolds were cut. Then, inner structure picture was taken by a Field Emission Scanning Electron Microscope (FE-SEM) (JSM-6701F, JEOL) after platinum coating. Scaffolds with cells were fixed with 4% paraformaldehyde (Polyscience) for 1 h, then washed in 0.1M Cacodylate / 2.5% sucrose and dehydrated with ethanol solution in sereies of 70%, 90% and 100%. Finally, hexamethyldisilazane (HMDS) (Daejung chemical) was introduced to cleaned samples for 1 h and observed with FE-SEM.

2.5 Cell culture

Human tonsil-derived mesenchymal stem cells (hTMSCs) (P6) were isolated as previously described with informed consent from patients at the Department of

Otorhinolaryngology Head and Neck Surgery, Ewha Woman's University Medical Center (EWUMC, Seoul, Korea)[9]. TMSCs were cultured in DMEM with 10%, 100 U/ml antibiotic-antimycotic and 100 U/ml penicillin-streptomycin at 37℃ with 5% CO2. Passage 4 cells were used for transfection plasmid containing gene encoding vascular endothelial growth factor (VEGF). After transfection, hTMSCs were cultured with growth medium containing DMEM supplemented with 10% FBS, 1% Pen-Strep, 1% L-glutamine, and 1% anti-anti up to 2 days then, used as *in vivo* samples.

Human umbilical vein endothelial cells (HUVECs) were seeded on 100mm tissue culture plate coated with gelatin (Millipore, Ultrapure water with 0.1% gelatin) ,for 30 minutes, and maintained in endothelial cell basal medium-2 (EBM-2; Lonza) supplemented with EGM^{TM}-2 except hydrocortisone. To get rid of remaining non-adherent cells medium was exchanged 24h after the initial plating and was exchanged every two days

2.6 Cell attachment and proliferation

For quantitative measuring of cell attachment rate on the scaffolds, Quant-iT^{TM} PicoGreen dsDNA Assay Kit (P11496, Invitrogen) was used. First, 5×10^5 cells seeded on each PEG, PEG-HA5 and PEG-HA10 scaffolds. After 3 hours, the cell seeded scaffolds were digested mechanically in papain solution which was composed of 25mg papain type III (Worthington) and 1.58mg cysteine in PBE buffer at 60℃ for 16 h. DNA amount was quantitatively measured with PicoGreen solution by Infinite M200pro (TECAN) for fluorescence spectroscopy. For cell proliferation rate measurements, AlamarBlue® cell viability reagent (Cat No. DAL1025) was used. AlamarBlue reagemt was added to cells

containing scaffold within the medium in the ratio of 1 to 10. After 3 h incubation at 37℃, chemical reduction of AlamarBlue® reagent / medium mixture was measured by using fluorescence spectroscopy (λ_{ex} = 550nm, λ_{em} = 600nm) by Infinite M200pro (TECAN).

2.7 Cell viability assay and morphological analysis

Cell viability in cryogels was confirmed utilizing live/dead cell viability/cytotoxicity kit (Molecular Probes; L-3224) that contains calcein-AM (for alive cells) and ethidium homodimer-1 (EthD-1, for dead cells) after 24 hours of cell seeding on each types of scaffolds. Then image was obtained using LSM 720 confocal microscope (Zeiss). For cellular morphological analysis, cells were fixed with 4% paraformaldehyde, washed with PBS and cut into 5-mm section. After permeabilization with 0.1% Triton X-100 for 30 min, sectioned samples were stained Alexa Fluor 488 Phalloidin in a ratio of 1:150 (A12379, Life Technologies) for 2 h and with 4¢6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) in a ratio of 1:200 for 5 min. Images were observed using LSM 720 confocal microscope.

2.8 Transfection

Human tonsil derived mesenchymal stem cells (TMSCs) were seeded 1×10^4 cells/cm^2 on a 100mm culture dish two days before transfection. Neon electroporation system (Invitrogen Inc.) was used to deliver pDNA encoding VEGFA (RG229636, Origene) to hTMSCs. 2×10^6 cells were washed and centrifuged two times with PBS and re-suspended with 100 μ L electroporation buffer (R buffer). VEGFA pDNA was mixed with hTMSCs in the proportion of 7.5 μg to $1x10^6$ cells in cell buffer mixture. Electroporation condition was at 1400 V and 1 pulses with pulse width 30 ms.

2.9 Real-time PCR analysis

The Samples were collected at certain time point weekly. Total RNAs were extracted from samples with Trizol method. cDNA was constructed by using TOPscript™ Reverse Transcriptase Kit (Enzynomics) according to the manufacturer's instruction. Real-time PCR was performed using TOPreal™ qPCR 2X premix (Enzynomics) and ABI StepOnePlus™ Real-Time PCR system (Applied Biosystems, USA). The expression level of genes of interes was determined by $-2^{\Delta\Delta Ct}$ method as previously described [17]., whereby the target was normalized to the endogenous reference (GAPDH). PCR was done three times for each sample. Sequences of each PCR primer are listed in supplementary table 1.

2.10 FACS analysis of transfected hTMSCs

After transfection, hTMSCs were cultured for next 48 hours. After that the cells were washed with PBS twice, then detached, collected with centrifugation and re-suspended in PBS. Cell / PBS solution was filtered with 35 μm cell-strainer (352235, BD Falcon). The GFP signal standard was set with normal hTMSCs as negative GFP signals, and transfected cells with positive GFP signals were sorted by FACS Aria II (BD Biosciences, San Diego, CA, USA).

2.11 *In vitro* angiogenesis analysis

200ul of chilled BD Matrigel™ matrix (BD Biosciences, BD Cat no. 354234) to every well

were added 24-well. Before cell seeding, plate was incubated for 20 minutes in incubator to make Matrigel gelated. 10×10^4 HUVECs per well were seeded onto Matrigel layer within well plate. Then they were incubated with EBM-2 containing 5% FBS or supplemented with PBS or Normal hTMSCs or VEGF hTMSCs or PEG-HA cryogels containing VEGF hTMSCs inside Transwell inserts with 8 μm microporous membrane. After 36 hours, the degree of tube formation of each group was analyzed by measuring covered area of tube networks at random chosen 3 spots of microscopic sight with Image J tools. Mice aortic rings were obtained from female BALB/c-nu mice at 6 weeks of age. Mice aorta was cut into several 1 mm segments. They was put in the middle of Matrigel in 24-well plate (Corning Incorporated) coated with 100ul of Matrigel at each upper and lower part. Then, PBS or Normal hTMSCs or VEGF hTMSCs or PEG-HA cryogels containing VEGF hTMSCs with endothelial cell basal medium-2 (EBM-2) without growth factors were introduced to the Transwell. Samples were incubated at 37℃ for 3 days. Samples were then photographed using EVOS (Life Technology).

2.12 Hindlimb ischemia model

All surgery procedures were approved by "Seoul National University Animal Care Committee (protocol #SNU-130226-2). For experiment, female BALB/c-nu mice age of 6 weeks (16-20g body weight; OrientBio) were anesthetized using zoletil and rompun. After skin removal, the femoral artery including branches was ligated with 6-0 silk (Ailee). As following step, all of the above arteries and external iliac artery were also ligated. Then, femoral artery was removed from its proximal origin as a branch of the external iliac artery to where it bifurcates into the popliteal and saphenous arteries. After surgery, mice were randomly assigned to five experimental groups (n = 5 for each group). No treatment was done to the

normal group. Ischemic induced mice were treated with injection of PBS or Normal hTMSCs or VEGF hTMSCs or PEG-HA cryogels containing VEGF hTMSCs (2 million cells per mouse). To quantitatively measure degree of ischemic induction, blood perfusion rate of post ischemia was evaluated by Laser-Doppler Flowmeter (Moor LDI, Moor instruments Ltd., Devon, UK). Measurement was done every week during 4 weeks and analyzed with moorLDI™-PC-software.

2.13 Histology & Immunohistochemistry3

Normal and ischemic limb Specimens were harvest after 4 weeks after hindlimb ischemia induction. Samples were fixed in 4% paraformaldehyde, dehydrated with 75%, 90%, 95%, 100% graded ethanol in series, and xylene. Treated samples were then embedded in paraffin. Specimens were cut into 4-μm sections. The sections were stained with hematoxylin & eosin (H&E) solution to examine the degree of tissue inflammation, muscle degradation, and vessel generation. Images were collected using CKS41 microscope (Olympus). To visualize micro vessels of samples, sections were stained with CD-31 (Novus Biologicals) and counterstained with 4, 6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Section samples were then photographed using EVOS (Life Technology).

2.14 statistical analysis

Data are expressed as mean± standard deviation. Statistical analysis was performed by the analysis of variance (ANOVA). P value less than 0.05 was considered statistically significant value.

3. Results

3.1 Hydrophobic substrate confirmation

To create hydrophobic surface substrate, we coated bare glass plate with PFDMA. a piece of Glass substrate was conformally coated with hydrophobic polymer film, 1H, 1H, 2H, 2H-perfluorodecyl methacrylate (PFDMA) via iCVD. X-ray photoelectron spectroscopy (XPS) survey scan spectra showed that control plate have only O1s, Na1s and Si2p peaks. But after PFDMA coating with iCVD, the F1s peak appeared with significant intensity. Also, O1s and si2p peaks disappeared completely compared to its original state, indicating that hydrophobic PFDMA polymers covered surface successfully (Fig. 1a). Then, exact quantity of atom was confirmed. After PFDMA deposition, O1s and Si2p atomic concentration decreased from each 58.54%, 27.37% to 5.12%, 0%. However, F1s increased from 0% to 64.85% (Fig. 1b). PFDMA substrate has high contact angle around 150° (148.34 ± 3.42°) compared to normal glass which has low contact angle (81.24 ± 2.78°) (Fig. 1c,d). This indicates that hydrophobicity was successfully introduced to bare glass substrate.

3.2 Effect of HA addition into PEG cryogels on scaffold structure, cell proliferation, attachment and viability.

In this studies cryogels with 0.05% (w/v) and 0.25% (w/v) of N,N,N',N' - tetramethylethylenediamine (TEMED) were characterized and utilized. The cryogels with 0.05% (w/v) of TEMED showed better porosity and swelling ratio (Supplementary Fig. 2). Therefore, we used 0.05% (w/v) of TEMED in every following experiments. Three SEM images showed

inner shape of each types (Fig. 1e). Addition of HA into PEGDA didn`t cause any changes in overall inner structure. PEGDA with 5% HA groups (PEG-HA 5) and PEGDA with 10% HA groups (PEG-HA 10) keep original shaped (upper low). At more magnified images (lower low), increasing of HA concentration didn`t hinder formation of macroporous interconnected pore morphology. Therefore, pore size still reserved (Fig. 1e). Moreover, HA enhanced initial cell attachment to cryogels. Compared with PEGDA cryogels itself (32.45 ± 1.02%), high cell attachment efficiency was measured at PEG-HA 5 (42.23 ± 1.35%) and PEG-HA 10 (46.75 ± 4.35%) (Fig. 1f). HA addition effects on cell proliferation was also confirmed. All the experiment data were normalized with cell number of initial seeding. In PEGDA cryogels, relative cell proliferation was 1.37 ± 0.07. However, PEG-HA 5 and PEG-HA 10 showed increased cell proliferation as 1.79 ± 0.11 and 1.94 ± 0.06. Insertion of HA into PEGDA increased cell proliferation rate (Fig. 1g). Furthermore, in order to precisely analyze viability of cells within scaffold Live/dead assay was performed. Fluorescence images of each three different groups showed that cells were barely died. Cells in three types of scaffolds were successfully survived (Fig. 2a). Quantified result indicated that the viability was maintained over 95% in all cryogels conditions (Fig. 2b). DAPI-phalloidin staining showed that hTMSCs were successfully attached and stretched in PEG-HA10 scaffold (Fig. 2c).

3.3 Enhanced expression of VEGF and other angiogenic gene of hTMSCs and HUVECs *in vitro*

To explore the angiogenic effect of VEGF pDNA transfection, cells had been analyzed since 2 days after transfection. In this assay we used VEGF pDNA tagged with green fluorescence protein (GFP) in order to easily check whether gene of interest was successfully

delivered into cells or not. Cells which had VEGF pDNA showed green fluorescence while negative control cells showed no fluorescence signal (Fig. 3a). Average efficiency of transfection quantified with FACS Aria II was measured to be 42.0% (Fig. 3b). Following transfection, the angiogenic gene expression level (endothelial receptors, anti-apoptotic factors, and adhesion molecules) was significantly increased in hTMSCs (Fig. 3c). Quantitative real-time polymerase chain reaction (RT-PCR) analysis explained that the expression of anti-apoptotic factors (PI3K and Akt-1) was increased ($p<0.001$) in VEGF transfected hTMSCs, compared with no transfection. VEGF transfection also upregulated ($p<0.001$) the expression level of endothelial receptors (Tie-2) and adhesion molecule (VCAM-1). Transgene (VEGF) expression level in hTMSCs was sustained up to 10 days after transfection (Fig. 4a). From two days after transgene transfection, VEGF expression was significantly boosted ($p<0.01$) in transfected hTMSCs, compared with no transfection (Fig. 4a). Also, it was confirmed that VEGF secretion level was not decreased in scaffolds. There was no hindrance to releasing VEGF inside cryogels. The maintenance period of VEGF expression of hTMSCs up to 1.5 week. This could make a contribution to improving hTMSCs viability at early time point after transplantation into ischemic induced site. To figure out actual effect of transplanted hTMSCs on endothelial cells, HUVECs were co-cultured with VEGF hTMSCs inside Transwell inserts. RT-PCR analysis was performed with co-cultured HUVECs. RT-PCR result analysis showed that the gene expression level of anti-apoptotic factors (PI3K and Akt-1), endothelial receptors (Tie-2) and adhesion molecule (VCAM-1) was increased ($p<0.001$) in VEGF transfected hTMSCs, compared with no transfection. However, fold increase of each gene level was slightly reduced compared with directly transfected hTMSCs. This suggests that VEGF released from hTMSCs facilitated the angiogenic differentiation of HUVECs (Fig. 4b).

3.4 The effect of VEGF hTMSCs on endothelial cell migration, angiogenesis of aorta ring and tube formation

3.4.1 Influence on cell migration

In order to examine the angiogenic effects of the VEGF hTMSCs on HUVECs, HUVEC migration assay was performed under addition of VEGF hTMSCs. We set negative control (EBM with 5% FBS), positive control (normal hTMSCs and VEGF hTMSCs without scaffolds) and experiment groups (VEGF hTMSCs with PEG-HA5 or PEG-HA10). HUVECs were plated at 24 well plate coated with gelatin. Cell seeding density was 2.5×10^5. Cells were cultured for 3 days until the confluent state was reached. Then, narrow scratch (wound) was introduced to hTMSCs monolayer by scraping with a 1ml micropipette tip. As following step, PBS washing removed cell debris from scratching. After that, incubation was proceeded in EBM-2 with 5% FBS, with each negative control, positive control and conditional groups. Cell migration was examined after 10 hours incubation. The negative control group and positive control group (normal cells) had no significant influence. However, not only VEGF hTMSCs itself, but also VEGF hTMSCs with PEG-HA10 facilitated ($p<0.001$) endothelial cell migration. In addition, VEGF hTMSCs with PEG-HA 5 group also presented positive effect ($p<0.01$). PEG-HA10 group showed enhanced cell migration compared with PEG-HA5 ($p<0.05$). This suggests that HA concentration played meaningful role at migration. Degree of cell migration was measured as the relative area covered by cells compared to the initial opening. Quantified analysis was performed using image J software (Fig. 5a, b).

3.4.2 Effect on angiogenesis of aorta ring and tube formation

In order to assess the angiogenic induction of the PEG-HA cryogels, capillary tube formation *in vitro* and vessel induction ability *ex vivo* was performed. We set negative control (EBM with 5% FBS), positive control (normal hTMSCs and VEGF hTMSCs without scaffolds) and experiment groups (VEGF hTMSCs with PEG-HA5 or PEG-HA10). HUVEC cells ($5x10^5$ cells/well) were seeded onto Matrigel-coated plates and incubated under the each condition. At negative control groups and Normal cell groups, minimal tube formation was observed. VEGF hTMSCs with PEG-HA10 showed comparable positive tube formation inductivity ($p<0.001$) to VEGF hTMSCs monolayer. In addition, VEGF hTMSCs with PEG-HA 5 group also presented positive effect ($p<0.01$). Moreover, Degree of tube formation was increased as the result of HA concentration, in a dose-dependent manner in VEGF hTMSCs with PEG-HA5 or PEG-HA10 group. PEG-HA10 group showed enhanced cell migration ($p<0.05$) compared with PEG-HA5 (Fig. 6a, b, c). We further examined angiogenic effect of our system using the endothelial cell sprouting from mouse aortic rings. As shown in Fig. 6d, the sprouts of endothelial cells from mice aorta showed different morphologies depend on the experimental groups. Tendency of degree of micro-vessel sprouting was similar to those of tube formation assay. Numerous sprouts and endothelial networks from the aortic ring were displayed in the experimental group treated with the VEGF hTMSCs with PEG-HA10, and VEGF hTMSCs monolayer ($P<0.01$). Likewise, endothelial sprouting was enhanced as the result of HA content, in a dose-dependent manner in VEGF hTMSCs with PEG-HA5 or PEG-HA10 group (Fig. 6e).

3.5 Rate of blood flow perfusion at *in vivo* hindlimb ischemia mice model

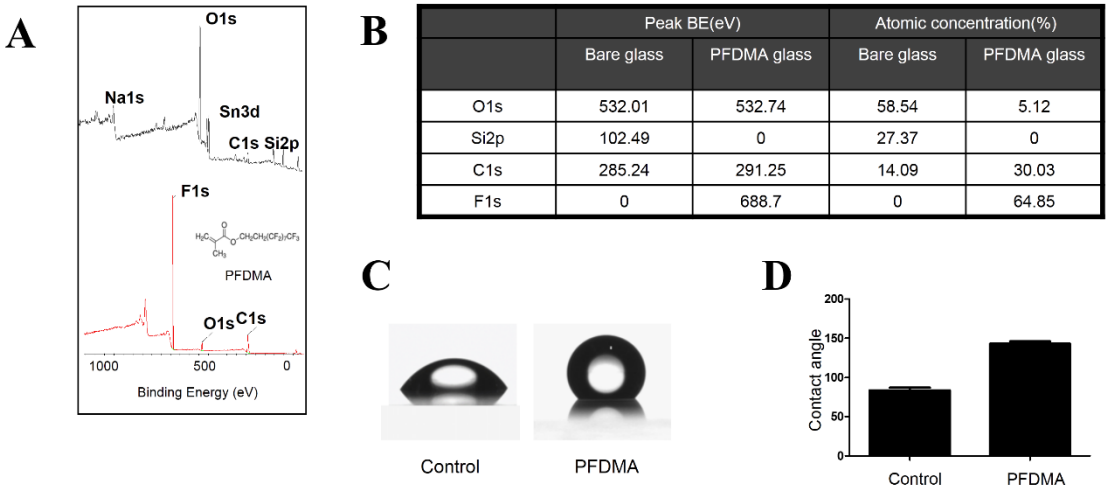
In order to assess the potential of our system as therapeutic method, hindlimb ischemia induced mice were used as *in vivo* model. Rate of blood flow perfusion was evaluated using

Laser Doppler imaging system. Right after ischemic limb induction, mice were intramuscularly treated with negative control (PBS), positive control (normal hTMSCs and VEGF hTMSCs without scaffolds) and experiment groups (VEGF hTMSCs with PEG-HA5 or PEG-HA10). The blood flow recovery of ischemic limb was calculated weekly as the ratio of ischemic leg (left side) perfusion to normal leg (right side) perfusion in same animal for 4 weeks. As shown in Fig. 7a, blood flow recovery was mainly proceeded in two groups: VEGF hTMSCs with PEG-HA5 or PEG-HA10. Mice injected with VEGF hTMSCs without carriers only showed limited positive effect. The rest of control groups (PBS and normal hTMSCs only) showed a no recovery of blood flow to the ischemic limb, resulting in complete limb loss (Fig. 7a). The statistical diagram of rate of blood flow perfusion seems quite in accord with the laser Doppler image (Fig. 7b). The VEGF hTMSCs with PEG-HA5 or PEG-HA10 led to a markedly superior perfusion recovery, conflicting with *in vitro* results in that experimental groups (VEGF hTMSCs with PEG-HA5 or PEG-HA10) displayed better therapeutic influence than just transfected hTMSCs without scaffold. The VEGF hTMSCs with cryogel improved ($p<0.001$) ischemic limb salvage, compared with either negative control (PBS), positive control (normal hTMSCs and VEGF hTMSCs without scaffolds (Fig. 7c).

3.7. Vessel formation and muscle degradation analysis via immunohistochemistry

For assessing vessel formation and muscle degeneration, hematoxylin & eosin (H&E) staining was carried out in the specimens extracted at 4 weeks after transplantation. This confirmed that transplanted VEGF hTMSCs led to improved micro vessel regeneration which eventually prevented thigh muscles from necrosis damage from ischemia induction. As shown in upper low of Fig. 7d, H&E staining of the control group (PBS treatment and normal cells

only) displayed massive muscle degradation at the ischemia induced site. On the other hand, VEGF hTMSCs with cryogels protected limb from perfect necrosis (Fig. 7d). To examine whether micro vessels were newly formed at the injected site, CD31 immunohistochemistry was performed in the harvested thigh muscles. Histological images was depicted in Fig. 7C, suggesting improved angiogenesis. Specimens treated with only PBS or normal hTMSCs only demonstrated low micro-vessel formation. In contrast, improved angiogenesis was evaluated in the VEGF hTMSCs with PEG-HA groups. Measured capillary formation (lower low; Fig. 7d) using the CD31 IHC showed quite similar tendency in accord with those as in Fig. 7d upper low. Implantation with the VEGF hTMSCs with PEG-HA5 or PEG-HA10 led to the capillary generation with longer length and larger diameters. These results, the presence of newly made vessels in the ischemic induction area, indicate that our new system facilitated angiogenesis.



	Peak BE(eV)		Atomic concentration(%)	
	Bare glass	PFDMA glass	Bare glass	PFDMA glass
O1s	532.01	532.74	58.54	5.12
Si2p	102.49	0	27.37	0
C1s	285.24	291.25	14.09	30.03
F1s	0	688.7	0	64.85

Fig. 1

Characterization of the iCVD processed hydrophobic substrate and micro cryogel system. (a) XPS survey scan spectra of bare substrate (top) and PFDMA-coated hydrophobic substrate (bottom). Inlet; chemical structure of PFDMA. (b) Table showing quantified amount of each atoms on substrate surface. (c) Contact angle images and (d) Graph of water dropped onto each substrates.

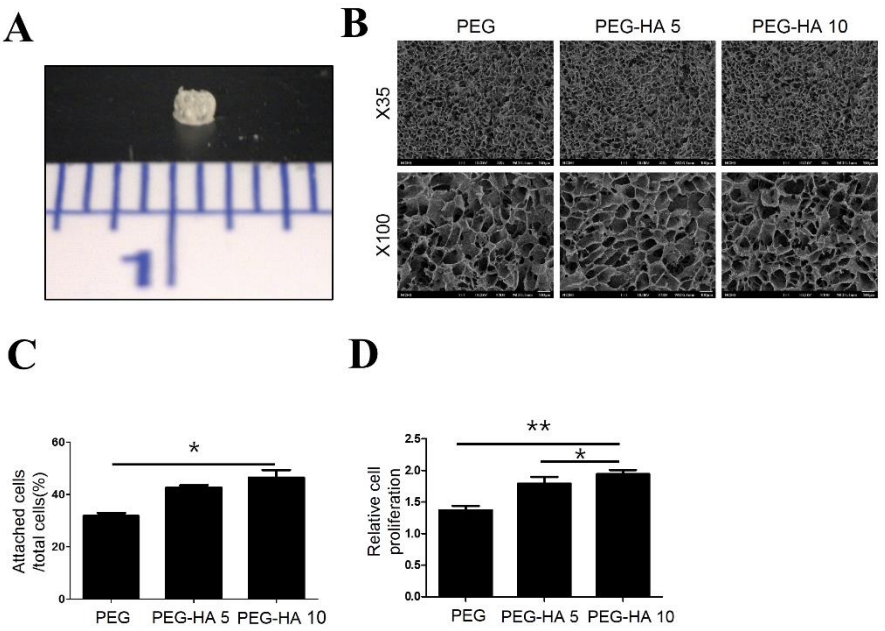


Fig. 2

(a) Field emission scanning electron microscope images representing inner structure of each cryogel groups. (b) Number of attached cells per initial seeding number of cells. (c) Relative cell proliferation rate of cells inside cryogels.

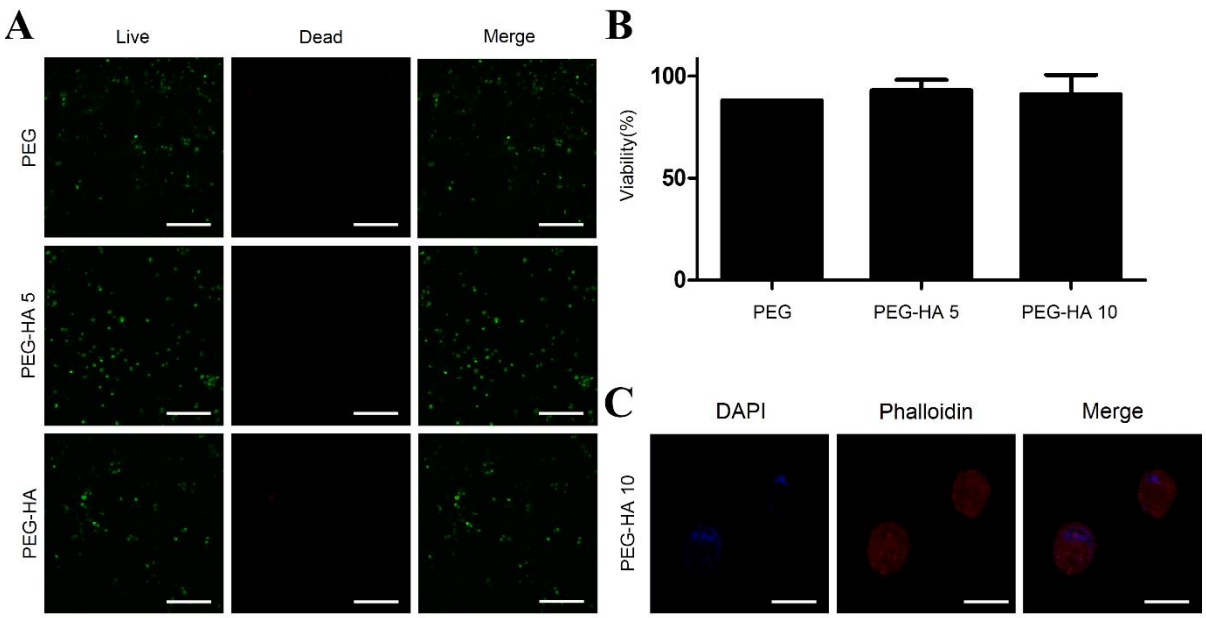


Fig. 3

(a) Analysis of hTMSCs viability in HA-added PEGDA cryogels. Viability test was performed using live/dead viability cytotoxicity kit. Dead cells were stained with Ethd-1 and alive cells were stained with Calcein AM. Scale bar = 200 mm. (b) Viability was quantified by measuring the ratio of live cell to total cell number. Standard deviation was represented by Error bars on the mean for n = 3. (c) Morphological analysis of hTMSCs in HA-added PEGDA cryogels. Each group was stained using DAPI and phalloidin to observe actin cytoskeleton and nucleus. Scale bar = 100 mm.

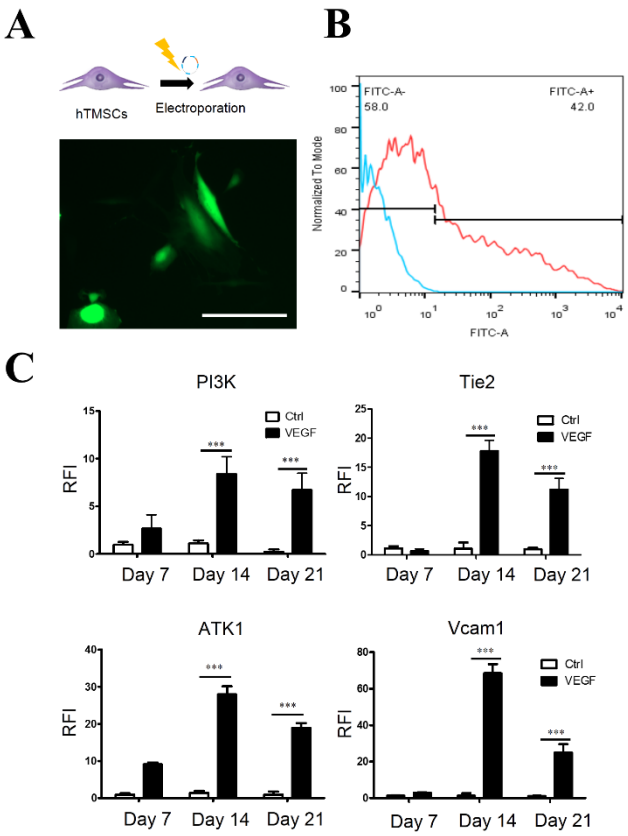


Fig. 4

Effect of VEGF transfection on hTMSCs. (a) Upper picture; graphical scheme of electroporation. Lower image; GFP fluorescence image of transfected hTMSCs (b) Flow cytometry data analysis for GFP tagged VEGF pDNA delivery via electroporation. Transfection efficiency was measured as green fluorescent protein-positive live cells per total live cells. (c) Quantitative real-time polymerase chain reaction (RT-PCR) analysis of transfected hTMSCs for PI3K, Tie-2, Akt-1 and VCAM-1 ($n = 3$; *** means $p < 0.001$; compared to control groups). The relative expression of each molecule was normalized to GAPDH group.

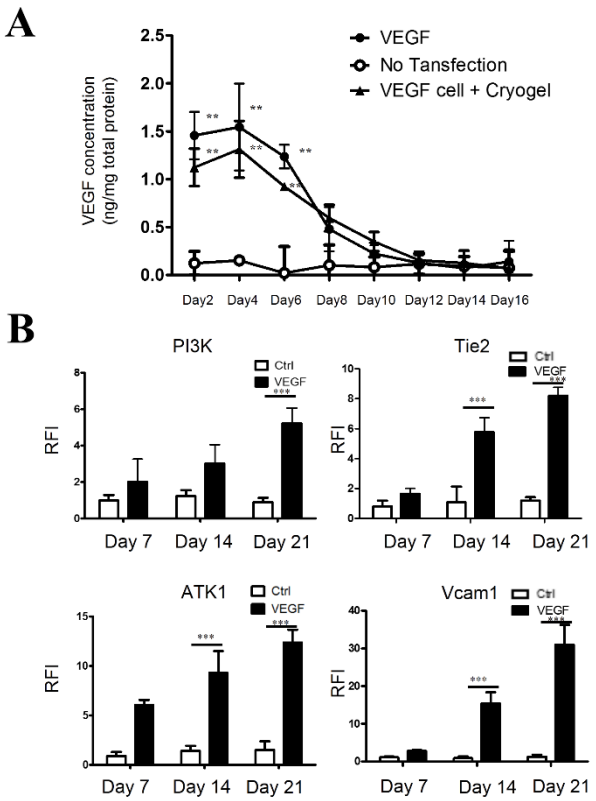


Fig. 5

Influence of VEGF secreted from transfected hTMSCs on HUVECs. (a) ELISA for demonstrating the duration of VEGF secretion in hTMSCs (2, 4, 6, 8, 10, 12, 14 and 16 days) after transfection (n = 3; ** means $p < 0.01$; compared to no transfection group).

(b) Quantitative real-time polymerase chain reaction (RT-PCR) analysis of HUVECs for PI3K, Tie-2, Akt-1 and VCAM-1 (n = 3; *** means $p < 0.001$; compared to control groups). The relative expression of each molecule was normalized to GAPDH group.

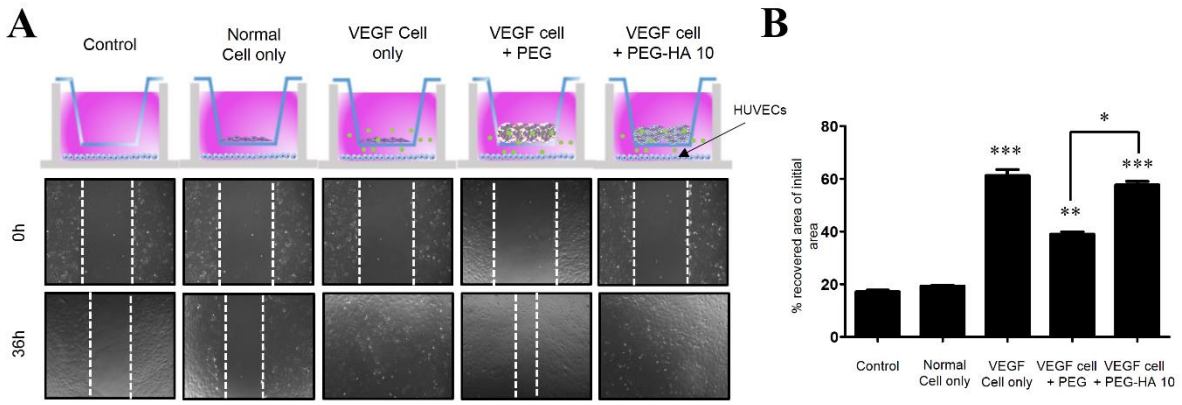


Fig. 6

Response of HUVECs migration of *in vitro* under hTMSCs with PEG-HA cryogels. (a) Upper picture; graphical scheme of wound healing assay. Lower images; representative images of wounding recovery migration at two time point (0 and 36 hours). (b) Migration of HUVECs was measured in response negative control (PBS), positive control (normal hTMSCs and VEGF hTMSCs without scaffolds) and experiment groups (VEGF hTMSCs with PEG-HA5 or PEG-HA10). Degree of wound healing migration was quantified as the recovered area to the total area inside the reference line (n = 3; *, **, *** mean each p <0.05, p <0.01 and p < 0.001; compared to other groups).

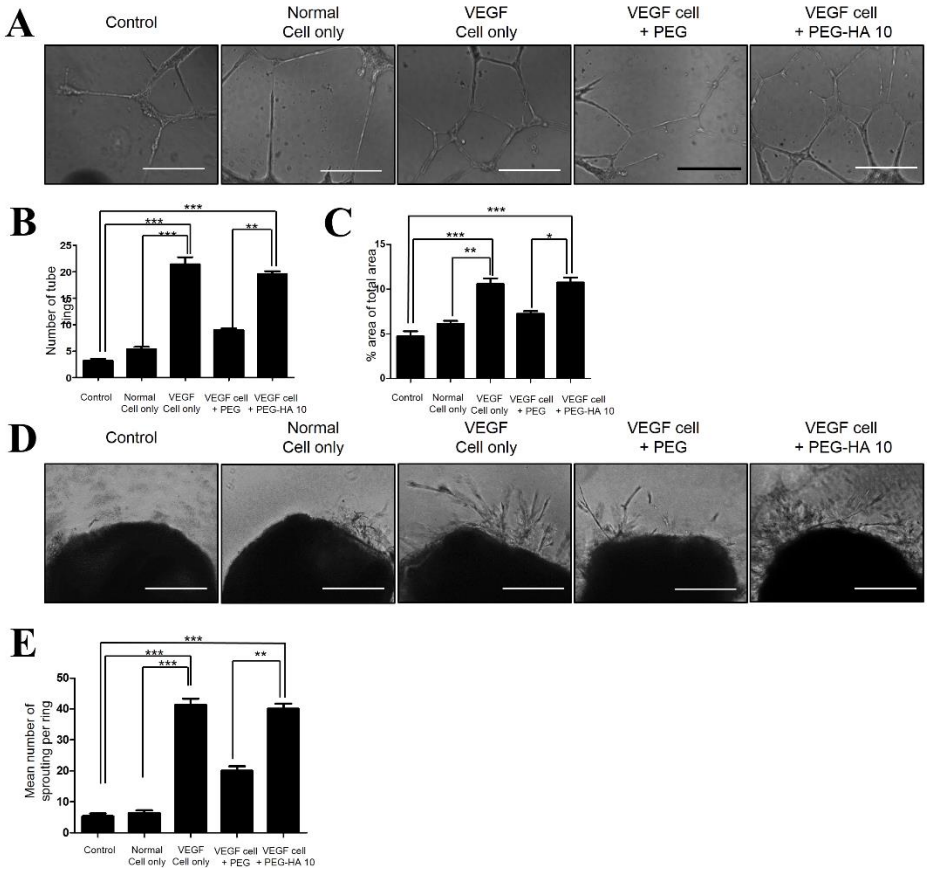


Fig. 7

(a) Images representing tube formation assays of HUVECs *in vitro* under hTMSCs with PEG-HA cryogels. Scale bar = 200 μm. (b, c) Tubule structure of HUVECs under negative control (PBS), positive control (normal hTMSCs and VEGF hTMSCs without scaffolds) and experiment groups (VEGF hTMSCs with PEG-HA5 or PEG-HA10. Tube formation was quantified by measuring number of tube in microscopic field at three different point. The area of tubular structure was measured as tubular area to total area.

Ex vivo aorta sprouting assay was performed with aortic rings, harvested from mice. Aorta was cultured in the middle matrigel coated on Transwell system. The hTMSCs with PEG-HA cryogels were added to upper panel. Scale bar = 200 μm. These samples were cultured for 3 days. (d) Images of angiogenic sprouting of aorta. Scale bar =100 μm (e) Scored values represented the number of vessels sprouted from each rings (n = 3; **, *** mean each p <0.01

and $p < 0.001$; compared to other groups).

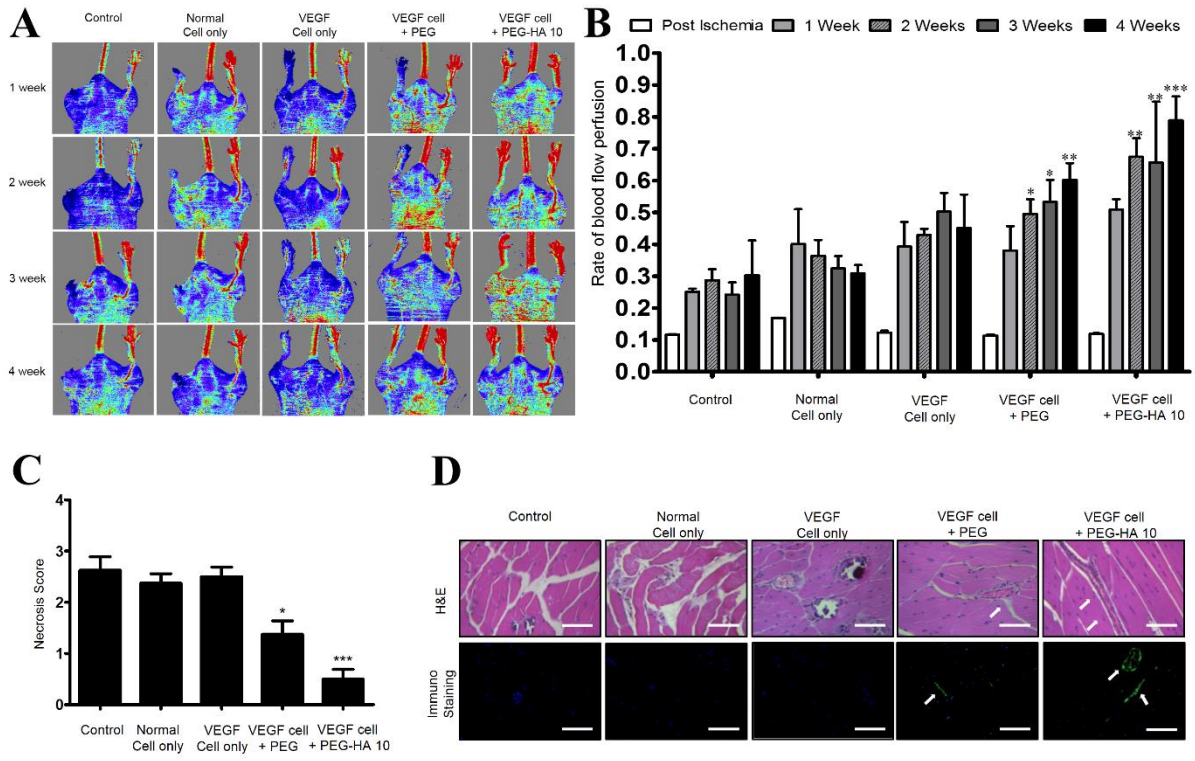


Fig. 8

Therapeutic effects on mice blood flow perfusion and ischemic limb salvage. Negative control (PBS), positive control (normal hTMSCs and VEGF hTMSCs without scaffolds) and experiment groups (VEGF hTMSCs with PEG-HA5 or PEG-HA10) were injected into the ischemic induced mice limb right after hindlimb ischemia induction. Representative images and blood flow perfusion was weekly for 4 weeks. (a) Blood flow perfusion images of mice. (b) Ratio of blood flow perfusion was measured as the ischemic limb (left leg) over the normal limb (right leg) at every week for 4weeks (* means $p < .05$). (c) Necrosis score after 4 weeks (n= 5 ; **, *** mean each $p < .01$ and $p < .001$). (d) Histological images of ischemic induced limbs and normal limb after 4 weeks. Upper low; H&E staining. White arrows represent neovascularized micro vessels. Lower low; immunofluorescence staining of DAPI and CD31 after 4 weeks. White arrows represent neovascularized micro vessels. Scale bar = 100 μm

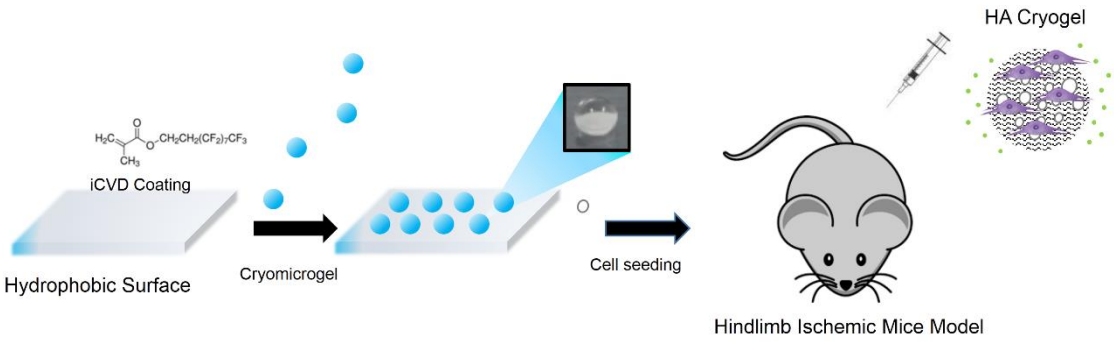


Fig. 9

Experimental Scheme

4. Discussion

Neovascularization inducing cell therapy has promising potential for a variety of field such as osteogenic field, and other medical application including rheumatoid arthritis and cancer [18-23]. Recently, cell therapy has taken center stage that it could enhance angiogenic processes using genetically engineered stem cells expressing vascular endothelial growth factors [1, 24-28]. This induced the formation of capillary structures with a significant increase in total area and rate of blood flow [7, 11].

A numerous and various types of cell lines, for instance endothelial progenitor cells, cardiac stem cells, natural killer cells, bone marrow cells and dendritic cells, have been investigated in clinical revascularization trials [8]. It is quite certain that stem cell based therapies held promise as a useful treatment for dealing with a variety of diseases, including diabetes, hyperlipidemia and even cancer. This is, indeed, because of stem cell`s multipotent ability and other released factors that plays important role in cure through the neovascularization and inhibition of apoptosis.

However, it was reported that cell-based therapies and direct angiogenic factor delivery methods have several limitation as applicative medical therapy. The biggest shortcoming was short duration of directly delivered protein factors due to host immune system as well as proteolytic degradation. Thus, it strongly requires new methods for protein delivery like the gene recombination with nonviral vectors such as transfection [28]. Moreover, cell carriers caused tissue damage to host from the injection bolus and undesirable cell viability after injection or implantation [29]. To overcome such hindrances, new cell carriers like cryogels has been started to be used. In accordance with previous study, hyaluronic acid (HA) can

facilitate cell attachment and migration into the scaffold due to its high water retention and intrinsic swelling property [12]. Moreover, it is specifically adhesive to CD44 surface marker [30]. Therefore, HA alone or cross-linked with other materials has been widely utilized as cell delivery carriers. In addition, human tonsil derived stem cells (hTMSCs) not only express CD44 surface marker but also has multipotent ability which is not fully understood [9]. Thus, using hTMSCs with HA added cryogels shed a new light on improved cell carrier system for angiogenesis.

In the present study, we fabricated PEGDA cryogels containing HA without any mold but only with hydrophobic substrate. Spherical shape was obtained by introducing hydrophilic materials onto hydrophobic surface (Fig. 1e). HA contents didn`t affect shape and structure of cryogels. Macroporous interconnected pore morphology was also preserved. This suggest that HA addition didn`t changed basic physical characteristics of PEGDA. Moreover, HA improved cell attachment and proliferation within cryogels (Fig. 1f, g). HA might provide cellular attachment site. So, it was observed that hTMSCs could possibly survive and proliferate more easily than bare PEGDA. This was also confirmed with DAPI-phalloidin staining which demonstrated that cells were tightly attached and stretched in PEG-HA (Fig. 2c). In addition, PEGDA-HA cryogels didn`t show significant cell cytotoxicity in our study (Fig. 2a).

Furthermore, we have demonstrated that our new system VEGF hTMSCs with PEG-HA successfully enhanced angiogenic gene expression of HUVECs, endothelial cell migration, tube formation, *ex vivo* mice aorta ring sprouting and mouse blood flow recovery *in vivo* (Figs. 4-7). First, expression of transfected transgene, VEGF facilitated anti-apoptotic factors in hTMSCs. The expression level of this signaling factors (PI3K and Akt-1) was upregulated after VEGF delivery (Fig. 3c). PI3K and Akt-1 signal pathway which could started by VEGF might

can restrain apoptosis [31]. After injection, transplanted hTMSCs might be exposed to host immune system or harsh environment. This circumstance might led injected cells to apoptosis. However, injected cells are more likely survived because of not only our cryogel system but also anti-apoptotic gene upregulation due to transfection. Second, it has been reported that VEGF increased the expression level of endothelial receptors (Tie-2) and the signal pathway regulated by these receptors [28, 32]. The gene expression level of these receptors much enhanced in hTMSCs. Then, activation of these gene may promote cell proliferation of endothelial cells [28, 33]. We demonstrated that VEGF secretion was successfully occurred from transfected hTMSCs (Fig. 4a). Also, it is confirmed that released VEGF protein mediated angiogenic gene upregulation in HUVECs co-cultured with hTMSCs (Fig. 4b). The increased gene expression level of endothelial receptors might facilitate endothelial cell proliferation in ischemic site. Third, it was known that VEGF could upregulate gene expression level of adhesion molecules (VCAM-1) [34]. Based on our study described so far, it was shown that the expression level of VCAM-1 was increased in hTMSCs (Fig. 3c). Likewise, VEGF secretion from transplanted hTMSCs facilitate adhesion molecule expression in co-cultured HUVECs (Fig, 4b). This indicated that hTMSCs could influence the intrinsic endothelial cells in injected site which is usually ischemic region. The enhanced expression level of adhesion molecules in ECs may facilitate EC mobilization and incorporation into ischemic region [28, 34]. Improved gene expression level of these factors including anti-apoptotic gene, endothelial receptor and adhesion molecules assisted endothelial cell engraftment into original tissue. So significantly enhanced angiogenesis was observed in ischemia area (Fig. 7d).

We also demonstrated that VEGF hTMSCs with PEG-HA facilitated endothelial cell migration at wound site, tube formation of HUVECs, and angiogenic sprouting from mice aorta

in vitro (Fig. 5, 6). Also, intramuscular injection of VEGF hTMSCs with PEG-HA brought about restoration of blood flow perfusion, regeneration of micro-vessels and inhibition of muscle degeneration in ischemic limb (Fig. 7). We finally assure that our new VEGF hTMSCs with PEG-HA might provide a key to inhibiting tissue necrosis, inducing neovascularization and homing intrinsic ECs toward ischemic site. Migrated ECs might increase in number, and participate regeneration of new capillary in ischemic hindlimb. Facilitated neovascularization in ischemic tissue restrained tissue degeneration and fibrosis caused by ischemic induction (Fig. 7d) and consequently improved ischemic limb salvage (Fig. 7a).

All of figures described above, suggested that enhanced angiogenic ability was induced through not only direct cell injection but also paracrine effects. These results are in accord with information from previous studies including a variety of stem cells and endothelial protein [35]. We suggest that the hTMSCs might be released from cryogels and support new vessel formation. Of course, further assay and researches would be needed to precisely explain this suggestion. It was also reported that human mesenchymal stem cells (hMSCs) were delivered as encapsulated state in a microgel. And they could migrate through the scaffold [36]. We used hTMSC which is also one branch of hMSCs. So this fact support that our hypothesis can be true. Altogether, our new hTMSCs with PEG-HA system, which was manufactured with hydrophobic substrate made via iCVD technique, may demonstrate a meaningful curative potential as treatment for vascular structure related disease.

5. Conclusion

VEGF has been explained as signal mediating growth factor which contributes to many parts of angiogenic pathway. However, instability of injected materials has always been a limitation as medicinal use. Herein, we suggested new cell therapy as vascular endothelial growth factor (VEGF) transfected human tonsil derived mesenchymal stem cells (hTMSCs), which have multipotent ability itself, within micro-cryogel. Micro-cryogels were made with poly (ethylene glycol) (PEG) which is known as biocompatible and biodegradable. We introduced hydrogel solution onto strong hydrophobic substrate manufactured via initiated chemical vapor deposition (iCVD). Hydrogel, then formed a spherical shape because of its own hydrophilicity. Chemically cross-linked gels were harvested and incubated including VEGF hTMSCs. Transfection efficiency was maintained over 40%. Moreover, both the size of cryogels and a number of seeded cells inside them could be controlled. GFP tagged VEGF releasing was confirmed with fluorescence microscopy and then quantified with VEGF ELISA assay. Furthermore, culturing hTMSCs within cryogels not only prolonged VEGF secretion duration but also kept secretion stably over certain level. Therefore, we suggest that our newly developed micro cryogel system held promise as a new therapy for neovascularization.

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요약 (국문초록)

편도유래줄기세포와 크라이오젤을 통한 치료목적의 신혈관생성

말초 동맥 질환은 당뇨병 환자에게 가장 흔하게 일어나는 증상이다. 최근 신혈관 생성을 촉진하는 방법이 이에 대한 새로운 치료법으로 떠오르고 있다.

이에, 혈관표피성장인자를 넣은 인간편도유래 줄기세포를 기존에 존재하지 않던 새로운 전달체로 사용함으로써 전달한다. 히알루론산이 포함된 작은 폴리에틸렌글라이콜 기반의 크라이오젤을 전달체로 사용한다. 폴리에틸렌글랑이콜일 경우 생적합성을 가진 물질로 보고되어 있다. 화학적 증기 증착을 통해 생성된 소수성 표면을 가진 기판을 제조한다. 이 위에 하이드로젤 용액을 떨어뜨리면 그 친수성 성질로 인하여 어떠한 주물이 필요없이 3차원 구조의 구 형태를 띄게 된다. 그리고 화학적으로 겔화된 하인드로젤은 동결건조 된다. 그 뒤 혈관표피성장인자로 유전적으로 조작되어진 편도유래줄기세포는 만들어진 크라이오젤 안에 넣어져 전달된다. 유전물질전달 효율은 40 % 이상으로 유지되었다. 또한 클라이오젤의 크기는 조절가능하였다. 혈관표피성장인자의 분비는 일라이자를 통하여 확인되었다. 편도유래줄기세포는 유전자 조작 하에서 단순히 혈관표피성장인자의 분비 기간이 연장되었을 뿐만 아니라 그 분비 정도가 어느 수준으로 일정기간 유지되었다. 또한 크라이오젤 안에서 치료효과를 가짐을 하지허혈 모델 하에서 확인하였다. 이에 우리는 새롭게 만들어진 이 크라이오젤 시스템이 단순히 당뇨로 인하여 고통받고 있는 환자들 뿐만 아니라 또한 다른 혈관류 질환을 앓고 있는 환자들에게 새로운 희망을 줄 수 있는 치료법이라 생각한다.골 형성을 유도하기 위해서 혈관유도와 무기환경 조성

을 포함한 다양한 방법이 연구되어왔다. 골 형성 과정에 필요한 충분한 양의 영양분과 산소를 분화중인 세포에 공급한다는 점에서 신생혈관재생은 성공적인 골 형성을 위한 가장 중요한 요인 중 하나로 작용한다. 이러한 관점에서 인간혈관표피성장인자를 이용한 다양한 방법이 혈관형성을 유도하고 골 형성을 증가시킬 수 있다는 것을 보여주었다. 뿐만 아니라, 무기물질이 침착된 미세환경은 골화 과정을 안정화시킨다고 알려져있다. 하이드록시아파타이트와 베타삼인산칼슘은 생체내 골분화유도능을 증가시키기 위해 널리 사용되어져왔다. 최근에 인산칼슘의 한 상인 휘트로카이트가 골 조직공학에 응용될 수 있는 무기 생체재료로써 합성되었다. 이번 학위논문에서 연구의 목표는 골 형성을 위해 인간혈관표피성장인자와 휘트로카이트의 잠재적 효과를 알아보는 것이었다. 이번 연구는 인간혈관표피성장인자와 휘트로카이트가 인간편도유래중간엽줄기세포의 골 분화를 촉진한다는 것을 공동상승작용을 보여줌으로서 증명하였다.

주요어 : 인간편도유래 중간엽줄기세포, 줄기 세포 치료, 약물전달, 히알루론산, 휘트로카이트, 크라이오젤, 신혈관생성

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