



Enhanced angiogenic activity of dimethyloxallylglycine-treated canine adipose tissue-derived mesenchymal stem cells

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ABSTRACT. The paracrine function of mesenchymal stem cells (MSCs) during transplantation has been recently studied due to its poor differentiation ratio. Dimethyloxallylglycine (DMOG) has been used to promote angiogenesis in experimental animal models, however, comparable approaches for canine MSCs are not sufficient. In the present study, we assessed whether DMOG improves angiogenesis in canine adipose tissue-derived mesenchymal stem cells (cAT-MSCs). cAT-MSCs were treated with DMOG and their effect on angiogenesis was investigated by cell proliferation assay, western blotting, and tube formation assay. Dimethyloxallylglycine preconditioning enhanced the expression of vascular endothelial growth factor (VEGF) among pro-angiogenic factors in cAT-MSCs via hypoxia-inducible factor-1 α stabilization. Dimethyloxallylglycine primed-cAT-MSC-conditioned media increased angiogenesis in human umbilical vein endothelial cells. These results suggest that DMOG conditioning of cAT-MSCs augmented the secretion of VEGF, which acted as a prominent pro-angiogenic factor during angiogenesis. DMOG-primed cAT-MSCs may have the potential to induce beneficial effects in ischemic diseases in clinical trials.

KEY WORDS: angiogenesis, dimethyloxallylglycine, dog, mesenchymal stem cell, vascular endothelial growth factor

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As undifferentiated cells, mesenchymal stem cells (MSCs) display self-renewability and the multipotency to differentiate into diverse tissues. Mesenchymal stem cells play a vital role in restoring damaged tissues. They are derived from different sources, such as bone marrow, fat tissue, the umbilical cord, and dental pulp [18, 26, 28, 35]. The adipose tissue of female canines is easily acquired during spay surgery. Fat tissue has been demonstrated to be an abundant source of MSCs [3]. Mesenchymal stem cells have been used to explore the application of MSCs in cell-based therapy, and the potential of MSCs has been proven by a number of studies on chronic kidney disease, cardiomyopathy, ischemic limb, and neurologic diseases [10, 14, 20, 24].

Several studies have shown that millions to billions of MSCs can be administered in animal disease models via intravenous or intraperitoneal injections in order to investigate their therapeutic effects [31]. Previous studies have also indicated that MSC-treated groups demonstrated ameliorations in deteriorated tissue, however, the transdifferentiation or cell fusion was too low to determine the source of the improvements in the injured tissue [21, 25, 34]. Paracrine signaling via the secretion of anti-inflammatory cytokines, growth factors, and myriad cellular molecules by MSCs may be responsible for these therapeutic outcomes [8, 13, 19].

Culturing MSCs under hypoxic conditions may be useful in enhancing angiogenesis activity via the secretion of growth factors [11, 14]. However, it is difficult to simulate hypoxic conditions in clinical settings for stem cell treatments as it is expensive to set up and manage hypoxic facilities. The influence that several chemical molecules exert on cells under normoxic conditions is similar to that under hypoxic conditions. The role of these chemical compounds in regulating the angiogenesis pathway to enhance remedial capacity has been investigated [1].

Dimethyloxallylglycine (DMOG) is a 2-oxoglutarate analogue and a cell-permeable compound that acts as a competitive inhibitor of prolyl-4-hydroxylase. Even under normoxic conditions, the prodrug DMOG is able to hinder the activity of prolyl hydroxylase domain (PHD) enzymes and sequentially restrains the degradation of hypoxia-inducible factor-1 α (HIF-1 α). Additionally, HIF-1 α migrates from the cytoplasm to the nucleus, where HIF-1 α and constitutively-expressed HIF-1 β combine to form a HIF-1 heterodimer. This heterodimer is a transcription factor that boosts the activation of HIF-1 signaling and influences

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the expression of genes encoding proteins responsible for cell survival, angiogenesis, neurogenesis, proliferation, differentiation, migration, and energy metabolism [17, 22]. To date, several studies have focused on the beneficial effects of DMOG in preconditioned MSCs for clinical trials.

To the best of our knowledge, only a few studies have explored the changes in growth factor secretion from DMOG-preconditioned cAT-MSCs. The purpose of the present study was to investigate whether growth factor secretion and angiogenic capability are increased after DMOG preconditioning in cAT-MSCs.

MATERIALS AND METHODS

Cell culture and characterization of cAT-MSCs treated with or without DMOG

Adipose tissues were obtained from three healthy bitches (one-year old, weighing 8–9 kg) during sterilization surgery. The owners of the donors provided written consent for research use. The procedure was approved by the Institutional Animal Care and Use Committee of Seoul National University (approval number SNU-180514-1) and the protocol abided by the approved guidelines. The fat tissue was aseptically sampled from the abdominal fat, and cAT-MSCs were isolated and cultured as previously described [12, 29]. Before the cell experiments, both 0.5 mM DMOG (Thermo Fisher Scientific, Waltham, MA, U.S.A.)-treated cells for 24 hr and non-treated cells were characterized by flow cytometry for their capacity to express stem cell markers. The following antibodies were used: cluster of differentiation 29 fluorescein isothiocyanate (CD29-FITC), CD34-phycoerythrin (PE), CD73-PE (BD Biosciences, Franklin Lakes, NJ, U.S.A.), and CD45-FITC (eBiosciences, San Diego, CA, U.S.A.)-conjugated antibodies. The DMOG-preconditioned and non-treated cells were classified into defined populations using a BD FACSAria II system (BD Biosciences). The results were examined using FlowJo 7.6.5 software (Tree Star Inc., Ashland, OR, U.S.A.). Cellular differentiation was identified using PRIME-XV[®] Chondrogenic Differentiation Xeno-Free Serum-Free Medium (SFM), PRIME-XV[®] Osteogenic Differentiation Medium, and PRIME-XV[®] 4 Adipogenic Differentiation SFM (all obtained from Irvine Scientific, Santa Ana, CA, U.S.A.) according to the manufacturer's instructions. To analyze differentiation, Alizarin Red, Alcian Blue, and Oil Red O staining were performed for osteogenesis, chondrogenesis, and adipogenesis, respectively.

Cell proliferation assay

To determine the DMOG concentration at which cAT-MSCs proliferation is affected, based on the findings of previous studies, DMOG at a concentration of 0–1 mM was used [16, 33]. A total of 3×10^3 cAT-MSCs per well were plated in 96-well cell culture plates with 0–1 mM DMOG. After culturing the cAT-MSCs for 12, 24, 48, and 72 hr, respectively, 10 μ l of Cell Counting Kit-8 (CCK-8; Dongin LS, Seoul, Korea) dye solution was added to each well. After incubating for 1 hr, the absorbance of the solution was measured at 450 nm using a spectrophotometer (US/680 microplate reader; Bio-Rad, Hercules, CA, U.S.A.). The wells containing DMEM without cells were used as blanks. The results were calculated using the following formula: $OD_t/OD_0 \times 100\%$, where OD denotes optical density.

Western blot assay

Total protein from MSCs was extracted using PRO-PREP Protein Extraction Solution (Intron Biotechnology, Seoul, Korea). The protein concentration was determined using the Bio-Rad DC Protein Assay kit (Bio-Rad). Thirty micrograms of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Biotrace, Pall, NY, U.S.A.). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20, and then incubated with affinity-purified rabbit anti-HIF-1 α (1:500; Lifespan Biosciences, Seattle, WA, U.S.A.) at 4°C overnight, followed by incubation with secondary antibodies for 3 hr at room temperature. Immunoreactive bands were normalized to β -actin (1:1,000; Santa Cruz, Santa Cruz, CA, U.S.A.) and visualized using Supersignal West Pico PLUS Chemiluminescent substrate (Thermo Fisher Scientific).

RNA extraction, cDNA synthesis, and quantitative reverse transcription polymerase chain reaction

Total RNA from cAT-MSCs was extracted using the Easy-BLUE Total RNA Extraction kit (Intron Biotechnology, Sungnam, Korea). The concentration and purity of the RNA samples were determined using a spectrophotometer (Implen, Munich, Germany). The cDNA was synthesized using 1 μ g of total RNA with the CellScript All-in-One 5X First Strand cDNA Synthesis Master Mix (CellSafe, Suwon, Korea). The samples were evaluated in duplicates in 10 μ l of AMPIGENE quantitative polymerase chain reaction (qPCR) Green Mix Hi-ROX with SYBR Green dye (Enzo Life Sciences, Farmingdale, NY, U.S.A.) using 1 μ l of cDNA and 400 nM of the forward and reverse primers (BIONICS, Seoul, Korea). The cycling conditions were as follows: polymerase activation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 5 sec, and annealing at 60°C for 25 sec. The double delta Ct ($\Delta\Delta Ct$) method was used to confirm the relative mRNA expression in samples by normalizing with the expression of glyceraldehyde 3-phosphate dehydrogenase housekeeping gene, and the fold change was evaluated using the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers used in this study are listed in Table 1.

Preparation of DMOG-conditioned media of cAT-MSCs

cAT-MSCs (3×10^5 cells/well) were seeded in 6-well plates and cultured in high glucose DMEM (DMEM; PAN-Biotech, Aidenbach, Germany) containing 2% fetal bovine serum (PAN-Biotech), and 1% penicillin-streptomycin (PAN-Biotech) with 0 or 0.5 mM DMOG [25]. The conditioned media (CM) were harvested and centrifuged at $300 \times g$ for 5 min to remove cellular debris

Table 1. Primers used for qRT-PCR

Gene	Forward	Reverse
VEGF-A	GAATGCAGACCAAAGAAAAGATAGAG	GATCTTGTACAAACAAATGCTTTCTC
bFGF	ACTGGCTTCTAAATGTGTTACTGAC	TAGCAGACATTGGAAGAAAAAGTAT
HGF	AAATAAACATATCTGTGGAGGATCA	CAAGCTTCATAATCTTTCAAGTCTC
Ang-1	GTTGGAAAGAATATAAAATGGGTTT	ATTCTTAGTGTGTACTGCCTCTGAC
HIF-1 α	ATGATGGTGACATGATTTACATTTCT	GTATTCTGCTCTTTACCCTTTTTCAC
GAPDH	TATGACGACATCAAGAAGGTAGTGA	GTAGCCAAATTCATTGTCATACCAG

every 24 hr for 72 hr. The supernatant was collected and stored at -80°C until further analysis.

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay was performed to evaluate the paracrine ability of the cAT-MSCs. The concentration of canine VEGF in the CM obtained by a method as described above was analyzed using the canine VEGF ELISA kit (R&D Systems, Minneapolis, MN, U.S.A.), according to the manufacturer's instructions.

Tube formation assay

The tube formation assay was used to evaluate angiogenesis *in vitro*. Matrigel (Corning, Corning, NY, U.S.A.), thawed overnight at 4°C , was administered into the wells of a cold 48-well cell culture plate and incubated at 37°C for 1 hr. Matrigel formed a thin gel layer during incubation. Human umbilical vein endothelial cells (HUVECs; 6×10^4 cells/well) (Lonza, Basel, Switzerland) were seeded into each coated well. Conditioned medium was also added to each well. As a positive control, DMEM with recombinant human VEGF, whose concentration was recommended by HUVEC Culturing Guidelines, was used. After incubation at 37°C for 2 hr, tube formation was visualized under a microscope (Olympus, Tokyo, Japan). The degree of angiogenesis was analyzed using ImageJ software.

Statistical analyses

All data are presented as the mean \pm standard deviation (SD). The statistical significance of the mean values was compared using one-way analysis of variance (ANOVA) and Student's *t*-test using GraphPad Prism v.6.01 software (GraphPad Software Inc., San Diego, CA, U.S.A.). Results with *P*-values <0.05 were considered statistically significant.

RESULTS

Characterization of cAT-MSCs and effects of DMOG on phenotype markers of cAT-MSCs

The cAT-MSCs differentiated into adipogenic, osteogenic, and chondrogenic cells, as was confirmed by Oil Red O, Alizarin Red, and Alcian Blue staining, respectively (Fig. 1A). In addition, the surface markers CD29, CD73, CD34, and CD45 were used to evaluate whether the immunophenotypic characteristics of cAT-MSCs changed after DMOG treatment (Fig. 1B). The flow cytometry results showed high expression of CD29 and CD73, and the absence of expression of CD34 and CD45 in cAT-MSCs and DMOG-treated cAT-MSCs.

DMOG-treated cAT-MSC proliferation assessed by CCK-8

To evaluate the effect of DMOG on cAT-MSC proliferation, the cAT-MSCs were treated with various concentrations of DMOG for different time periods (Fig. 2). After treatment with DMOG, cell proliferation was suppressed at a dose of 1 mM after 12 hr. Furthermore, the proliferation ratio did not exhibit significant changes in cAT-MSCs treated with other concentrations of DMOG.

Effects of DMOG on the level of HIF-1 α in cAT-MSCs

To characterize the effect of DMOG on cAT-MSCs, the expression level of HIF-1 α was assessed by western blotting (Fig. 3). After culturing with 0, 0.1, and 0.5 mM DMOG for 24 hr, the 0.5 mM DMOG-preconditioned group presented the highest HIF-1 α expression level among the groups. The difference in HIF-1 α expression levels between the 0 and 0.1 mM DMOG-treated groups was not significant.

Effects of DMOG on mRNA expression of angiogenic factors in cAT-MSCs

The mRNA level of the angiogenic factors in the cAT-MSCs exposed to 0.5 mM DMOG was determined by qRT-PCR. The increase in VEGF expression was detected at 12 hr and reached the highest level at 24 hr (Fig. 4A). Moreover, the enhanced expression of VEGF lasted 72 hr. The expression level of basic fibroblast growth factor (bFGF) was not affected by DMOG until 48 hr, but decreased at 72 hr (Fig. 4B). However, the expression of hepatocyte growth factor (HGF) was not affected until 6 hr, but was restricted after 12 hr (Fig. 4C). The expression of angiopoietin-1 (Ang-1) decreased over time (Fig. 4D).

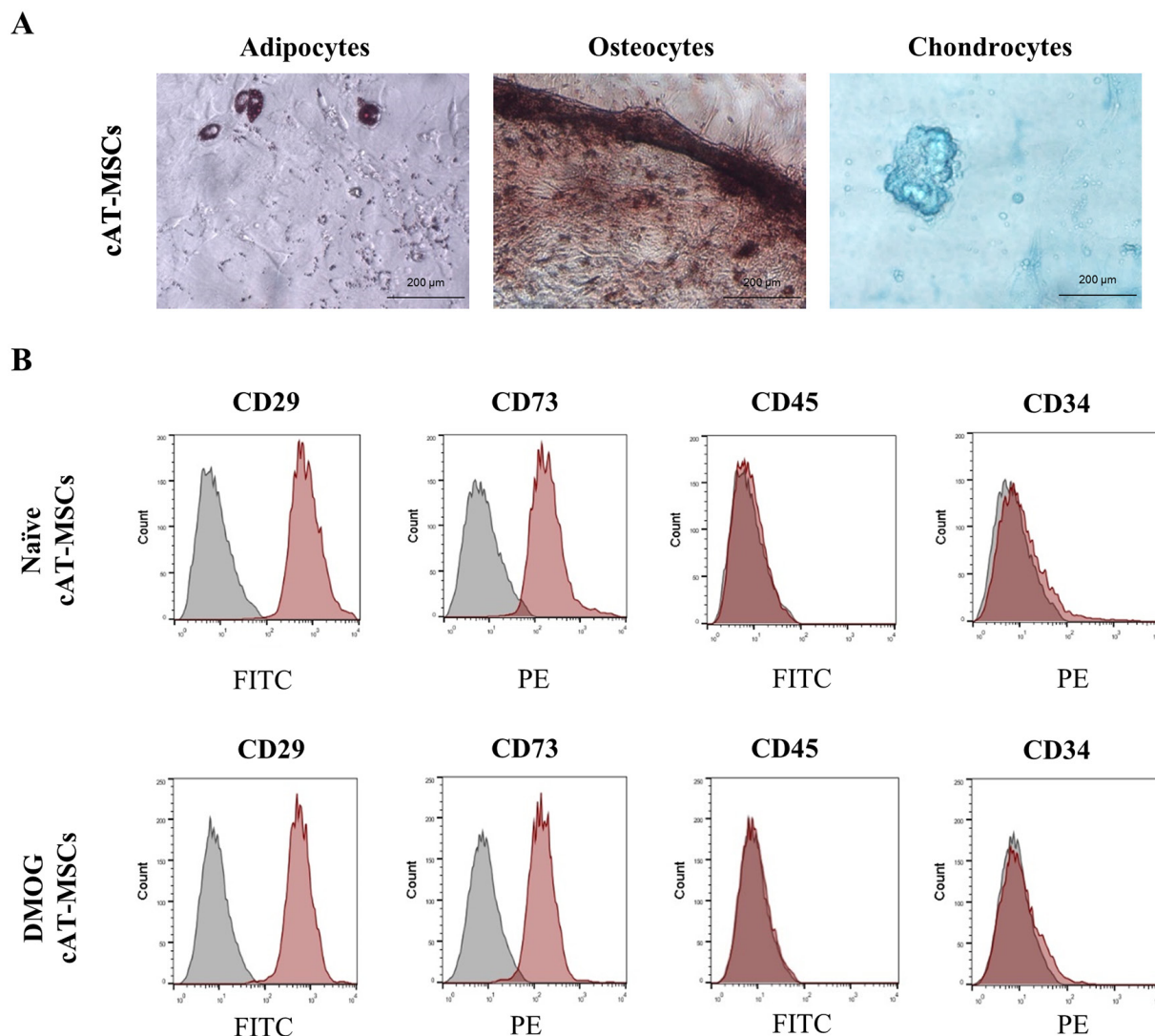


Fig. 1. Characterization of canine adipose tissue-derived mesenchymal stem cells (cAT-MSCs) and effects of dimethyloxalylglycine (DMOG) on phenotype markers. (A) cAT-MSCs cultured in specific differentiation media, differentiated into the adipogenic lineage (Oil Red O staining), osteogenic lineage (Alizarin Red S staining), and chondrogenic lineage (Safranin O staining). Original magnification, 100 \times . (B) Immunophenotypic analysis was conducted by flow cytometry for positive markers CD29 and CD73 and negative markers CD45 and CD34 in DMOG-treated or non-treated cAT-MSCs.

Effect of DMOG-CM on angiogenic activities

To investigate whether enhanced VEGF mRNA levels were translated into the corresponding enhancement of protein secretion, DMOG-CM collected at four time points was analyzed by ELISA (Fig. 5). The increase in VEGF levels started at 12 hr and reached a stable state at 24 hr. The concentration of VEGF in CM was higher than that of DMEM with recombinant VEGF, whose quantity was measured according to the HUVEC Culturing Guidelines. After culturing for 6 hr on Matrigel, the HUVECs cultured in DMOG-CM formed a higher number of tubes, and the total tube length and total branching points were higher than those incubated with DMEM and recombinant VEGF and untreated CM. This suggested that DMOG induced the production of VEGF, thus stimulating the formation of HUVEC capillary-like networks.

DISCUSSION

To the best of our knowledge, the present study is the first to demonstrate that DMOG can be applied to prime cAT-MSCs to enhance angiogenesis activity through the HIF-1 α pathway. Studies have reported that DMOG exerts no toxic effects on stem cell viability in various species [15, 33]. We compared the effects of several concentrations of DMOG on cAT-MSCs and found that cell proliferation was reduced 12 hr after treatment with 1 mM DMOG. However, the proliferation ratio was not significantly altered in cAT-MSCs treated with other concentrations of DMOG. Additionally, we determined the HIF-1 α activity after DMOG treatment, where 0.5 mM DMOG-preconditioned cAT-MSCs presented the highest expression levels of HIF-1 α . Moreover, the cAT-MSC

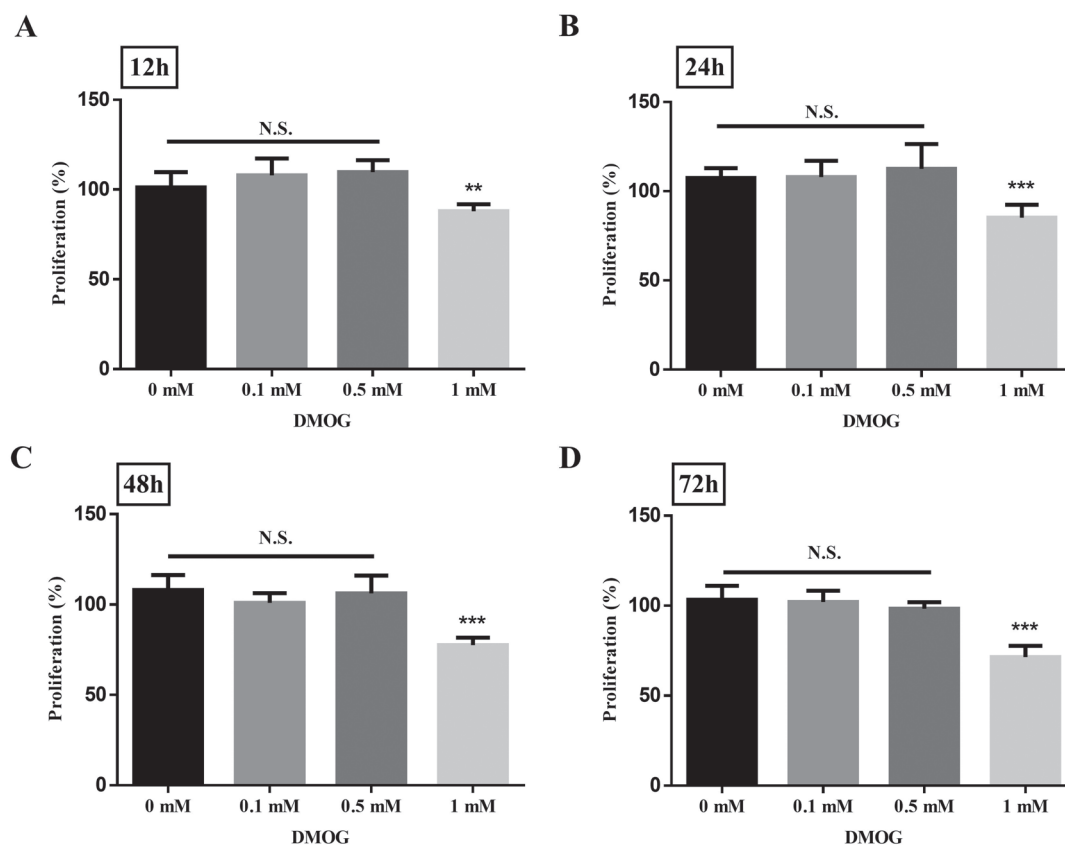


Fig. 2. Cell proliferation assay. After seeding in 96-well plates, cAT-MSCs (3×10^3 cells per well) were incubated for four different time periods (12, 24, 48, and 72 hr) and in four different dilutions of DMOG (0, 0.1, 0.5, and 1 mM). The results demonstrated the decreased survivability of cAT-MSCs only in 1 mM DMOG at every time point. The data are presented as the mean \pm SD of three independent experiments; *** $P < 0.0001$. N.S., not significant.

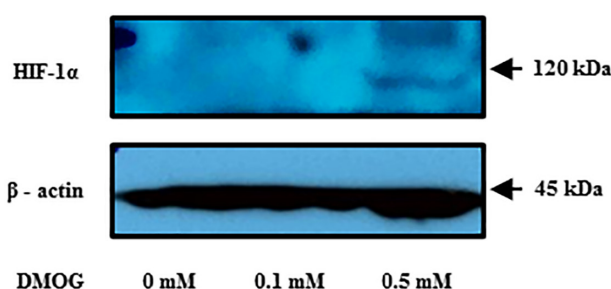


Fig. 3. Effect of DMOG on the expression of hypoxia-inducible factor-1 α (HIF-1 α) in cAT-MSCs. Western blotting showed that DMOG significantly enhanced the level of HIF-1 α in a DMOG concentration-dependent manner. The results were obtained from three independent experiments.

surface makers were found to not be altered in 0.5 mM DMOG-treated cAT-MSCs. Based on these results, we used 0.5 mM DMOG in further experiments with cAT-MSCs.

The results of the present study demonstrated that VEGF secretion in cAT-MSCs increased after exposure to DMOG. Although unaltered by DMOG treatment until 48 hr, the level of bFGF decreased at 72 hr. The level of HGF and Ang-1 was also evaluated as angiogenesis growth factors in stem cells [9, 23]. The results revealed that their expression levels decreased 12 hr after DMOG conditioning. To assess whether their expression levels increased during the early stages, their expression levels were measured at short intervals for the first 12 hr. The expression level of HGF was not affected by DMOG until 6 hr, however, it was restricted after 12 hr. The expression of Ang-1 declined with time. It is assumed that the expression of Ang-1 and HGF is influenced by the HIF-1 pathway and unknown signaling. This result suggests that DMOG is not suitable for the long-term treatment of cAT-MSCs. Previous studies have focused on the increased levels of paracrine factors in preconditioned stem cells [7, 30, 33]. It has been reported that the paracrine effects of angiogenic growth factors from stem cells are closely associated to the HIF-1 α pathway

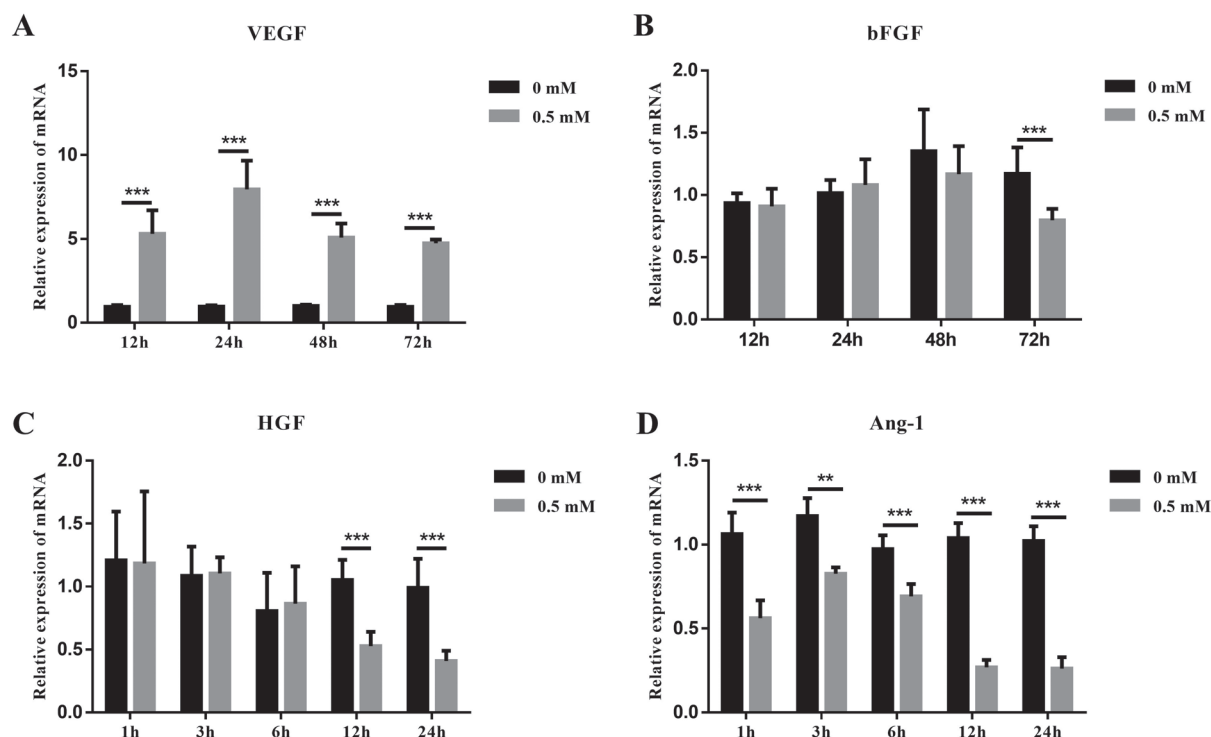


Fig. 4. The effects of DMOG on angiogenic-related gene expression in cAT-MSCs was determined by qRT-PCR. (A) The expression level of vascular endothelial growth factor (VEGF) was measured from 12 to 72 hr, and that of (B) basic fibroblast growth factor (bFGF), (C) hepatocyte growth factor (HGF), and (D) angiopoietin-1 (Ang-1) was determined from 12 to 24 hr. Each value represents the mean \pm SD of three independent experiments; ** P <0.01, and *** P <0.0001.

[27]. Studies have also indicated that VEGF and bFGF from stem cells increase angiogenesis via HIF-1 α stabilization during angiogenesis at the early stage [5, 32].

Recent studies have focused on the roles of VEGF after DMOG preconditioning of stem cells during angiogenesis [5, 15, 32]. Interestingly, the level of VEGF in cAT-MSCs treated with 0.5 mM DMOG for 24 hr increased considerably and lasted from 24 hr to 72 hr. The concentration of VEGF in 0.5 mM DMOG-treated cAT-MSC medium was higher than that in DMEM with recombinant VEGF, whose concentration was similar to that recommended by the manufacturer for culturing HUVECs. In the tube formation assay, the number of branches and tubes, as well as the length from HUVECs in cAT-MSC media containing 0.5 mM DMOG were superior to those of any other medium. Moreover, the VEGF concentration in 0.1 mM DMOG-treated cAT-MSC-CM was higher than that from non-DMOG treated cAT-MSC-CM, but lower than that from cAT-MSC medium supplemented with 0.5 mM DMOG (Supplementary Fig. 1). Based on the results of the previous study, it is evident that VEGF levels and tube forming ability are correlated in cAT-MSCs.

An enhanced secretion of angiogenic molecules could have a therapeutic potential against ischemic diseases. Several studies have indicated that ischemic symptoms are ameliorated by combinational usage of recombinant VEGFs, however, the improvement does not last long owing to their short half-lives [2, 6]. Moreover, gene therapies using overexpressing growth factors result in low therapeutic advantages owing to poor transgene expression [30]. Compared with these therapeutic regimens, stem-cell therapy is a promising treatment, as stem cells continue to secrete VEGF throughout their lifespan.

DMOG may enhance the survivability of stem cells under pathologic environments [4, 16, 32], although the improvement in cell survival was not evaluated in the present study. In addition, further studies are needed to investigate whether the VEGF inhibitor affects the angiogenesis activity of DMOG-primed cAT-MSCs. In light of the interaction between DMOG and cAT-MSCs, these results provide evidence for the further application of DMOG in the treatment of ischemic diseases in clinical trials.

In the present study, 0.5 mM DMOG did not alter the surface markers on cAT-MSCs or the proliferative ability of cAT-MSCs. As a biochemical, DMOG augmented the expression of HIF-1 α and enhanced the secretion of VEGF from cAT-MSCs. These findings demonstrated that DMOG-primed cAT-MSCs increased the secretion of VEGF, which acted as a pro-angiogenic factor, and stimulated angiogenesis.

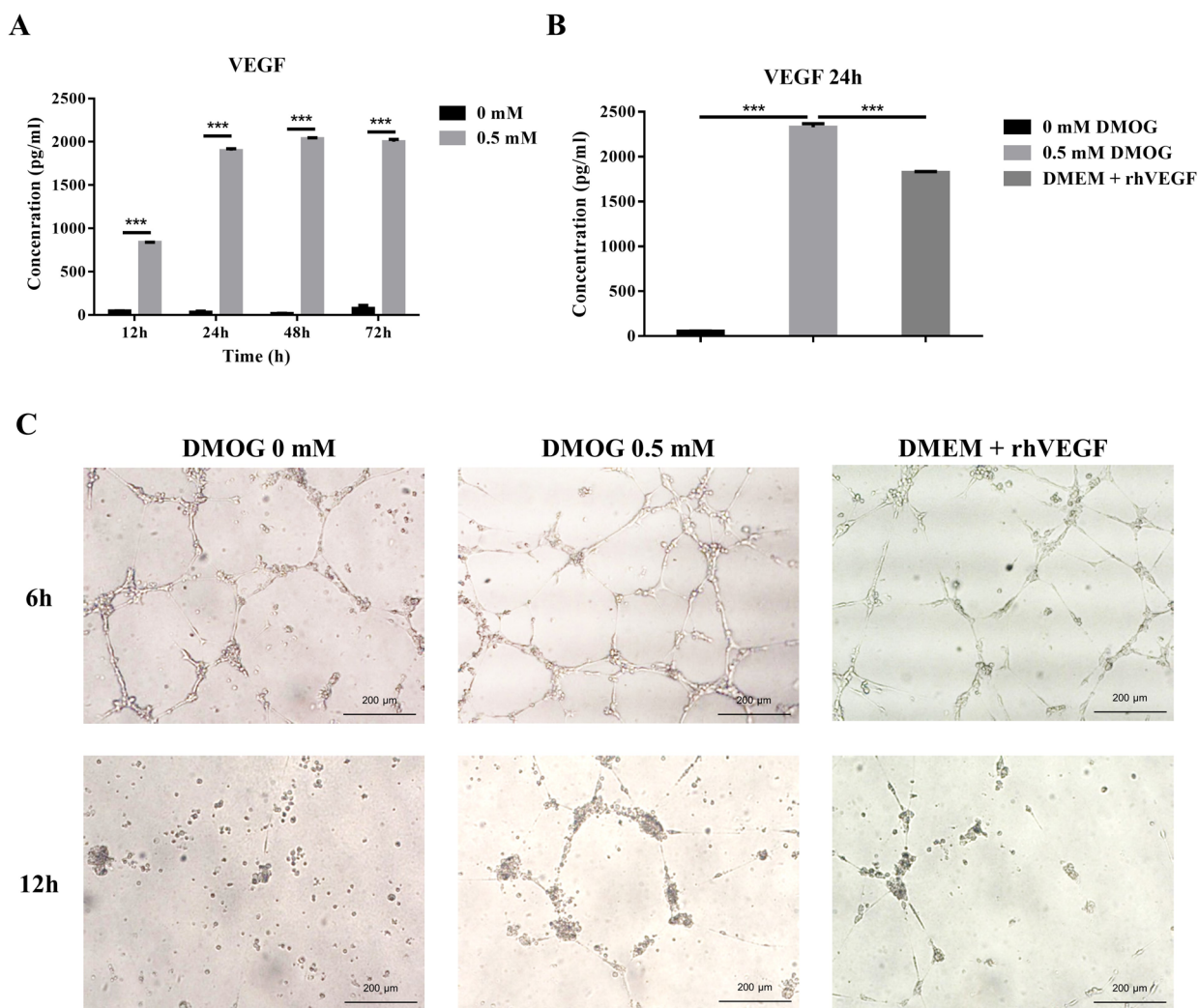


Fig. 5. The effects of DMOG on the secretion of VEGF from cAT-MSCs were analyzed by ELISA and tube formation assay. (A) The production of VEGF from DMOG-treated cAT-MSCs was quantified in a time-dependent manner and (B) was compared with that in different media. (C) Tube formation in human umbilical vein endothelial cells (HUVECs) was studied by culturing cAT-MSCs on Matrigel in DMEM with recombinant VEGF or two conditioned media. The results are presented as mean \pm SD of three independent experiments; *** P <0.0001.

CONFLICT OF INTEREST. There are no conflicts of interest to declare.

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