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| Original Article |

Dental Pulp Stem Cells derived Conditioned Medium Promotes Angiogenesis in Hindlimb Ischemia

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Abstract : Paracrine effects of mesenchymal stem cells (MSCs) have been suggested play an important role in the treatment of ischemic diseases. Dental pulp Stem cells (DPSCs) share many properties with MSCs. However, the beneficial effects of DPSCs on ischemic diseases remain to be elucidated. The present study, we found that DPSCs secreted higher levels angiogenic factors of VEGF, SDF-1, MCP-1 and PDGF-BB compared with AD-MSCs. We then investigated whether DP-CM can induce the migration of vascular smooth muscle cells (VSMCs) and human umbilical venous endothelial cells (HUVECs) *in vitro*. Under hypoxia, the apoptosis of HUVECs was inhibited while survival was improved by treatment of DP-CM. In a H_2O_2-induced cell death assay, DP-CM also significantly reduced HUVECs oxidative stress compare to control group. The tube formation assay demonstrated that the DP-CM group had a greater angiogenic potential than control medium. Results in the mouse model showed both the laser Doppler perfusion index and the relative number of CD31 positive microvessels to be significantly higher in the DP-CM group than in the control group [(77%±11%) vs. (45%±6%), and (6.2±1.1)/HPF vs. (2.3±0.3)/HPF]. In this way, the use of DP-CM may be a suitable means of treating ischemic diseases.

Key words: *Hindlimb ischemia; Dental pulp stem cells; HUVECs; Conditioned media; Angiogenesis*

1. Introduction

Peripheral arterial disease (PAD) is an atherosclerotic disease caused by insufficient blood flow to the lower extremities.[1] The traditional clinical stages of treating PAD are invasive interventions such as balloon angioplasty, stenting and surgical revascularization.[2] One novel approach to treating PAD involves stimulation of vascular repair mechanisms.[3] MSCs have been utilized for treatment of ischemic diseases.[4,5] However, despite their ability to replace and repair damaged tissue, the low survival rates of stem cells after transplantation limit their effects to the short term. The cells usually fall prey to hypoxia and inflammatory cytokines.[6,7] Accordingly, it has been hypothesized that paracrine factors from stem cells may provide more therapeutic benefits than those cells. The processes leading to angiogenesis involve molecules such as VEGF, PDGF-BB, SDF-1 and others. These molecules lead to HUVECs and VSMC migration, proliferation, vessel formation and maturation.[8-10] It has been shown that BM-MSCs conditioned media (MSC-CM) can lead to angiogenesis and regeneration of lung tissue.[11] Furthermore, high levels of proangiogenic cytokines in the BM-MSC-CM such as VEGF-a, IGF-1, PDGF-BB and bFGF recruit endothelial progenitor cells into the ischemic tissue thus enhancing angiogenesis.[12] Other study have demonstrated that adipose tissue derived mesenchymal stem cells (AD-MSCs) secrete cytokines and growth factors that stimulate angiogenesis, cell survival and activation of endogenous stem cells[13].

DPSCs isolated from milk teeth have been exhibited therapeutic potential in the treatment of ischemic diseases.[14-16] Dental pulp tissue embryonic origin from neural crests contains blood vessels and neuron precursor cells. These cells can differentiate into vascular cells and neurons very efficiently.[17] Previous studies have suggested that DPSCs have shown a high proliferation rate and exhibit more angiogenic potential than bone marrow mesenchymal stem cells (BM-MSCs) in defective

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tissues.[18,19] The reported angiogenic properties of DPSCs make them attractive for use in ischemic diseases. However, it is still remain uncertain whether DPSCs can exert their beneficial effects on the regeneration of damaged tissues via paracrine mechanisms involving secretion of soluble factors such as growth factors and cytokines. For this reason, in this study, we compared the levels of angiogenic factors secreted by DPSCs, AD-MSCs and fibroblast and found that DPSCs secrete relatively higher levels of variety of growth factors and cytokines into conditioned medium. The therapeutic benefits of DP-CM on hindlimb ischemia were investigated *in vitro* and *in vivo*. DP-CM was found to induce migration and tube formation in VSMCs and HUVECs very efficiently. It is here demonstrated that DP-CM also contributes directly to the protection of HUVECs from hypoxia and oxidative stress through expression of several anti-apoptotic soluble factors. DP-CM was found to alleviate hindlimb ischemia in a mouse model very effectively by consistently improving blood flow complete with angiogenesis. The present results suggest that DP-CM may provide another stem cell-based treatment for some kinds of ischemic diseases.

2. Material and Methods

2.1 Isolation and Culture of DPSCs

Human dental pulp samples were collected from donors with their consent at the Sichuan University Hospital of Stomatology. DPSCs were isolated as previously described.[20] Dental pulp was obtained after dental procedures and washed twice with phosphate-buffered saline (PBS; Gibco, Invitrogen, Grand Island, NY, USA). The pulp was cut into small pieces then digested with 3 mg/mL Collagenase I (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C and cultured in a 6-well plate (Costar, Corning, USA) at a density of 1×10^6 cells/cm^2 in a growth medium containing α-MEM (Invitrogen, Grand Island, NY, USA) supplemented with 10% (v/v) FBS (Gibco), 2 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco), 0.25 μg/mL Amphotericin B (Gibco). Non-adherent cells were removed by changing the medium after 2 days. Cells were passaged with trypsin at 90% confluence. The growth medium was changed every 3 days.

2.2 Differentiation

Osteogenic, chondrogenic and adipogenic differentiation were performed by using a StemPro Differentiation Kit (Gibco) according to the manufacturer's instructions, with some modifications. Briefly£¨for osteogenic differentiation, at 75% confluence MSCs were incubated in a 24-well plate with osteogenesis differentiation medium. The medium was changed every 3 days. On day 21, the cells were processed for Alizarin Red S staining (Sigma-Aldrich). For chondrogenic differentiation, cells were transferred into a 15 mL polypropylene tube and centrifuged at 200 g for 5 min to form a cell pellet at the bottom of the tube. After one day the medium was changed to chondrogenic differentiation medium, and the cell pellets were processed for Toluidine Blue staining (Sigma-Aldrich) after 21 days. For adipogenic differentiation MSCs were seeded in a 24-well plate at a density of 2×10^4 cells/cm^2. Cells were incubated for 22 days and fed adipogenesis differentiation medium every 3 days. Adipogenic differentiation was examined by Oil Red O staining (Sigma-Aldrich).

2.3 Phenotype Identification

Flow cytometry analysis was performed to examine the expression of the MSCs markers as previously described. DPSCs were cultured in the growth medium for 4 days at a density of 1×10^4 cells/cm^2 and harvested by trypsin. A Stemflow™ hMSC Analysis Kit (BD Biosciences, NY, USA) was used for a multicolor flow cytometry assay, containing pre-conjugated and pre-titrated cocktails markers (CD105 PerCP-Cy5.5/CD73/APC/CD90-FITC) negative markers (CD45/ CD34/CD11b/ CD19/HLA-DR-PE) and isotype controls. After washing with PBS, 1×10^6 cells were analyzed using a flow cytometer (FACSAria; BD Biosciences).

2.4 Preparation of DP-CM

To obtain DP-CM, DPSCs from 3 to 6 passages were seeded at 8000 cells/cm^2. At 80% confluence the cells were washed three times with PBS and the medium was replaced with serum-free DMEM (Gibco). After 72 h the medium was centrifuged and filtered through a 0.22-μm filter. Media were analyzed using ELISA and liquidchip assay that tested for the presence of 14 different cytokines and growth factors. They were then stored at -70°C until use. For *in vivo* assays the medium was concentrated 10-fold using an ultrafiltration membrane with a molecular weight cut-off of 3 kDa (Pall, NY, USA).

2.5 Endothelial tube Formation assay

Endothelial tube formation was performed as described previously with some modifications.[21] An *in vitro* angiogenesis assay kit (Millipore, Billerica, MA, USA) was used for the tube formation assay. Human umbilical vein endothelial cells (HUVECs) were incubated in a 24-well plate with Matrigel at 3×10^5 cells/well for 20 h in serum-free DMEM or DP-CM. Tubule junction, ring formed were counted for each condition in three randomly-selected fields. The tubule lengthc sums and

the average tubule diameter taken from three random fields of view in each well were analyzed using ImageJ software.

2.6 Migration Assay

Invasion chambers were used by 8 μm pore size transwell inserts (Corning, NY, USA) coated with 100 μl of Matrigel (BD Biosciences) diluted 1:25 (v/v) with DMEM. One hundred fifty microliters of CFSE (Dojindo, Tokyo, Japan) labeled VSMCs and HUVEC suspension in DMEM containing 1% BSA was added onto the upper chamber. Then 800 μL of DP-CM and DMEM was loaded into the lower chamber. Studies were performed 3 times with each group. After 16 h, cells remain on the upper chamber were scraped by cotton swab. Then the cells migrated to the lower chamber was determined using a fluorescent microscopy (DMI3000B, Leica, Wetzlar, Germany). Three fields per chamber were randomly selected.

2.7 Cell Survival and Apoptosis Assays

100 μL of HUVECs suspension in a 96-well plate (5000 cells/well). Cells were incubated under hypoxic conditions in a tri-gas incubator (MCO-19M, Sanyo, Tokyo, Japan), set at 37°C, 1% O_2, 5%CO_2, 94% N2 (Sanyo) and serum deprivation. Then 10 μl CCK-8 solution (Dojindo) was added to each well, and its absorbance was measured at 450 nm using a microplate reader (Multiskan, Thermo Scientific, Hudson, NH, USA) at 24, 48, and 72 h. The cells were then randomly sorted into two groups by serum-free DMEM and DP-CM.

To detect the cyto-protective effect of DP-CM, HUVECs were plated in a 6-well plate at a density of 1×10^5/well with DP-CM or DMEM. Then they were incubated in the tri-gas incubator 1% O_2, 5%CO_2, 94% N2 for 48 h. HUVECs incubated in 5% CO_2 with DMEM supplied with 10% FBS as negative control. DNase I (Beyotime, NanTong, China) were used to induce apoptosis of HUVECs as positive control. Apoptosis rate of HUVECs was detected by TUNEL assay kit (Beyotime). Briefly, HUVECs were xed with 4% paraformaldehyde (JHD, ShanTou, China) in PBS, rinsed with PBS twice, then permeabilized by 0.1% Triton X-100. The TUNEL-positive cells were imaged under a uorescent microscopy (Leica). The number of HUVECs was determined by Hoechst 33258 staining (Beyotime).

To determine the effects of DP-CM on oxidative stress induced HUVECs apoptosis, oxidation-induced cell death assay was performed. HUVECs were plated in six-well plates (Corning) and incubated in either DP-CM or DMEM and treated with 500 μM H_2O_2 (LDH) for 4 h to induce oxidative stress. For the detection of apoptotic cells, flow cytometry was performed using an Annexin V-FITC/7-AAD apoptosis detection kit (Biolegend). The data analyses were performed using flowJo software (Tree Star, Ashland, OR, USA).

2.8 Animal Model of Hindlimb Ischemia

All animal experiments were performed in accordance with the ethics committee of the West China Second University Hospital. Male 14-16 g C57BL/6 mice (Experimental Animal Center of Sichuan University) were anesthetized with 10% chlorohydrate (JHD) (0.1 mL/10 g). Hindlimb ischemia was created by surgically resectioning one femoral artery as described. Briefly, after sterilization with povidone-iodine, a part segment of the left femoral artery was exposed, then removed and ligated bilaterally. After suture and disinfection of the incision site, all animals were placed under a heat lamp for 30 min to restore their body temperature. Two days and five days after surgery, 0.3 ml concentrated DP-CM or serum-free DMEM was injected intramuscularly in the ischemic areas and nearby areas (0.05 mL/point). There were 6 mice in the DP-CM treatment group and 6 mice in the DMEM group.

2.9 Assessment of Blood Perfusion

A Laser Doppler perfusion imaging device was used to measure the blood flow of ischemic and non-ischemic limbs 24 days after the procedure (Moor Instruments, Axminster, Devon, UK). In the images, deep blue indicates low or no blood perfusion; whereas, Areas with the most perfusion are shown in red. After blood flow was scanned twice, the flow values of the ischemic and non-ischemic limbs were averaged. Then, blood flow perfusion recovery rates were compared between the ischemic and non-ischemic limbs.

2.10 Immunohistochemistry Analysis

Immunohistochemical analysis was performed according to the published protocol.[22] Muscle tissue sections from ischemic leg were fixed with 4% paraformaldehyde overnight and embedded in paraffin. Before analysis, the tissue sections were deparaffinized, rehydrated, and stained with antibody CD31 (Biolegend, San Diego, CA, USA) vascular endothelial cell markers. Finally, the sections were incubated with H_2O_2 and 3,3'-diaminobenzidine for 10 min. Five areas were randomly selected for each animal (control, n=6; DP-CM treatment, n=6), and the number of CD31-positive microvessels in each area was determined under a microscope (Leica).

2.11 Growth Factor Assays

To analyze the accumulative release of factors and cytokines by DPSCs, conditioned media were harvested for analyses using ELISA and liquidchip assay. The levels of IGF-1, VEGF,

bFGF, SDF-1, MCP-1 and TGF-β in unconcentrated DP-CM were measured using an ELISA kit (RayBiotech, Norcross, GA, USA). Briefly, 200 μl of DP-CM or serum-free DMEM were added to 96-well microplates that were coated with a monoclonal antibody specific to the factor of interest and incubated for 3 h. After washing with PBS, the antibody was added to each well, the microplates were incubated for 1 h, washed with wash buffer, and substrate solution was added. After 45 min stop solution was added. The concentration of cytokine or growth factor was calculated by measuring the absorbance at 450 nm using a microplate reader (Multiskan, Thermo). The levels of EGF, FGF-2, TGF-α, IL-10, PDGF-BB, IP-10, MCP-1, MIP-1a, and VEGF were detected using liquidchip kits according to the instructions (Human cytokine magnetic kit, Millipore). Conditioned medium derived from AD-MSCs and fibroblast was detected as a control.

2.12 Statistical Analysis

Data were expressed as mean±standard error of the mean. Statistical comparisons were performed using Student's *t*-test. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1 Characterization of DPSCs

Dental pulp stem cells were isolated from dental pulp by their ability to adhere to a plastic dish.[23] After 2-3 days, single cells and small colonies can be observed. After 2 weeks, larger colonies were observed in the primary culture and cells were made ready for passaging (Fig 1A). DPSCs were examined for MSC marker expression using flow cytometry. Phenotypical analysis showed cells to be positivity for CD105, CD73, CD90 (>95%) and negative for CD45, CD34, CD11b, CD19, HLA-DR (<2%) . This was found to be consistent with the MSCs standards issured by ISCT (Fig 2).[24]

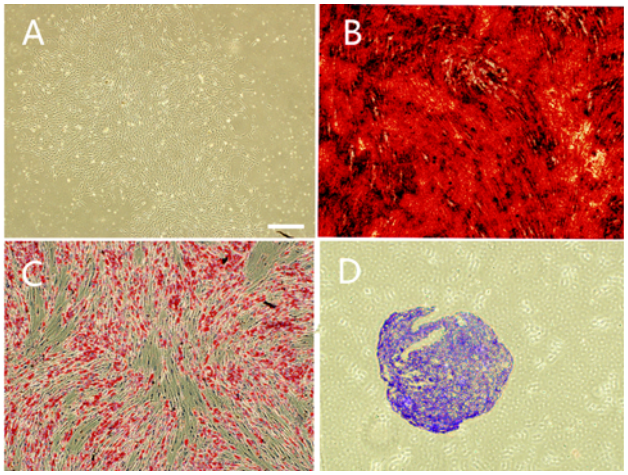

Figure 1. The morphology of DPSCs and the colony formed after 14 days (A). DPSCs were investigated for their osteogenic, adipogenic, and chondrogenic differentiation capacity to show their multilineage differentiation potential. After cells were cultured in the differentiation medium for 21 days, Osteogenic differentiation was examined by Alizarin Red staining (B). Adipogenic differentiation was examined by Oil Red O staining (C) and chondrogenic differentiation ability was examined by Toluidine Blue staining (D). Scale of bar in image = 100 μm.

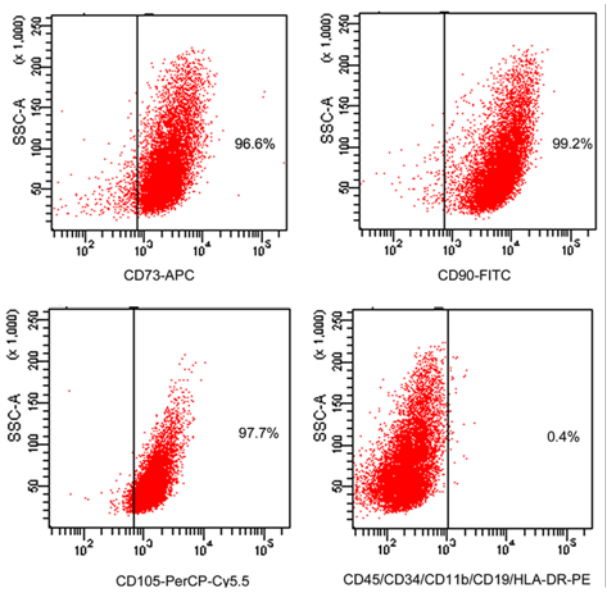

Figure 2. Analysis of the DPSCs marker expression by flow cytometry. Cells were detached using 0.25% trypisin and analyzed with a BD Stemflow hMSC Analysis Kit (n=3). The results indicate that DPSCs positive expression markers (CD105-PerCP-Cy5.5, CD73-APC, CD90-FITC) and negative expression markers (CD45/CD34/CD11b/CD19/HLA-DR PE) defined by the minimal criteria for MSC surface antigen phenotyping.

After 3 weeks of culture in appropriate differentiation medium, DPSCs were induced into osteoblasts, adipocytes and chondrocytes to identify their multilineage differentiation potential. Osteogenic differentiation culture demonstrated the capacity to form Alizarin Red positive condensed nodules with high levels of calcium (Fig 1B). Under the adipogenic conditions, DPSCs differentiate into lipid-containing adipocytes which were stained with Oil Red O (Fig 1C). Micromass cultures were used in chondrogenic differentiation. The cartilage nodules increased in size, when stained with toluidine blue, they showed proteoglycan subunits (Fig 1D).

3.2 DP-CM enhances Tube Formation *In Vitro*

Tube formation assay are used to study the ability of DP-CM

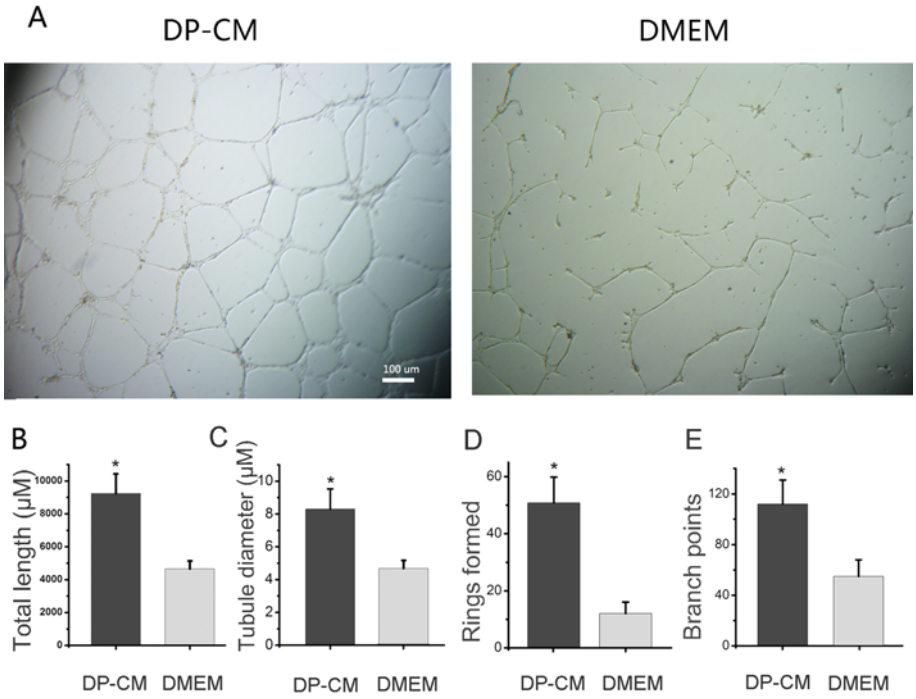

Figure 3. Matrigel endothelial tube formation assay. HUVECs were cultured for 20 h in 24-well plates coated with semi-solid EC matrix gel. Cells were cultured in the DP-CM (A left) or DMEM (A right) as control. The total tubule length (B), the average tubule diameter (C), Rings formed (D), and branch points (E) of the endothelial tube formation assay was measured. *$p < 0.05$ relative to the respective DMEM group according to a Student's T-test. Scale bar in image = 100 μm.

to cause HUVECs to form vessel-like structures. DP-CM showed a substantially more profound ability to form a complex tubular network after 4 h of incubation on the matrix than after the same amount of time in control medium (Fig 3A). Quantitative analysis demonstrated that HUVECs treated with DP-CM showed significant higher total tubule length, diameter than HUVECs treated with DMEM (Fig 3B, C). HUVECs grown in DMEM as negative control failed to form tubular structures, HUVECs as determined by the number of branch points (112±19 vs. 55±13), rings formed (51±9 vs. 12±4) (Fig 3D, E)

3.3 DP-CM Promoted VSMCs and HUVECs Migration

Blood vessels defects included a lack of recruitment of progenitor cells. Transwell migration was performed to evaluate the effects of DP-CM on the migration of VSMCs and HUVECs. More DP-CM than DMEM cells invaded the specified area (Fig 4A). Significant differences were observed between the two groups (P<0.05) (Fig 4B). The number of migrated cells in HUVEC/DMEM group was only one third of that in the HUVEC/DP-CM group (129±24 vs 43±7). There were more invasive cells in the VSMCs/DP-CM group (89±11) than in the VSMCs/DMEM group 30±6). This shows that DP-CM contains growth factors that contribute to accelerating

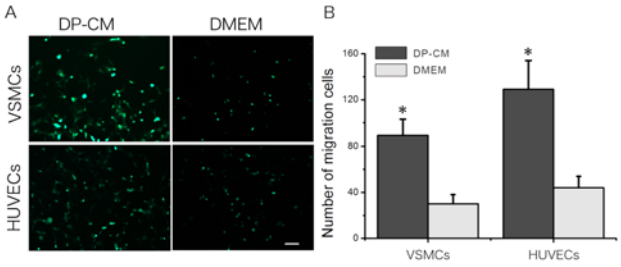

Figure 4. DP-CM enhanced VSMCs and HUVECs migration. (A) The chemotactic activity of DP-CM for VSMCs and HUVECs was determined. 5×10^4 VSMCs and HUVECs were collected during their passages 5 and allowed to migrate toward DP-CM or DMEM. Cells that invaded Matrigel were counted microscopically using three random 200× fields (n=3). (B) Graphical presentation of the quantified data displayed as number of migration cells. *$p < 0.05$ relative to the respective DMEM group. Bar Scale of bar in image =100 μm.

migration of VSMCs and HUVECs.

3.4 DP-CM Enhances HUVEC Survival under Hypoxia and Serum Starvation *In Vitro*

The capacity of DP-CM to prevent HUVECs from undergoing apoptosis under hypoxia and serum starvation conditions was

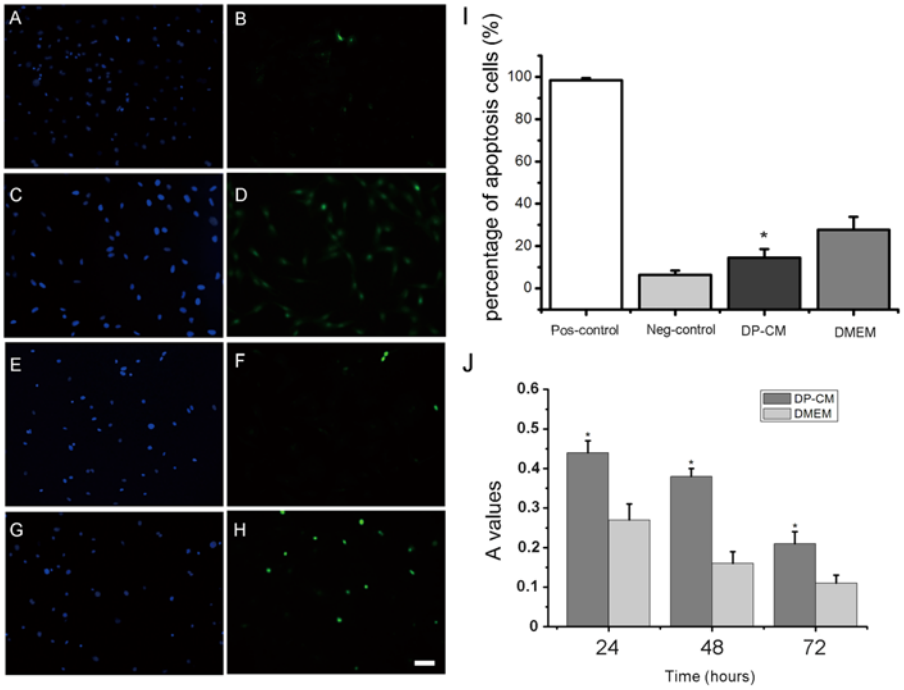

Figure 5. Protective effect of DP-CM on HUVECs under serum-free hypoxic conditions. TUNEL assay (B, D, F, H) and Hoechst 33258 (A, C, E, G) staining were performed to determine the proportion of apoptotic cells. Quantitative analysis of TUNEL positive cells content among groups. (A,B) Negative control showed 6.4±2% apoptosis rate, (C,D) Positive control showed 98.5±1% apoptosis rate. The apoptotic cells rate was significantly lower in DP-CM group than in DMEM group, 14.6±4% vs. 27.8±6% (P<0.05). HUVECs were cultured under serum deprivation and hypoxic conditions lasting 24, 48 and 72 h. The survival rates were determined by CCK8 (J). *p < 0.05 relative to the respective DMEM group. Scale bar in image = 100 μm.

analyzed using a CCK-8 assay and TUNEL assay. CCK-8 assay were performed after 24, 48 and 72 h of incubation under serum-free hypoxic conditions. The HUVECs survival rate in the DP-CM group (0.44±0.05) was significantly higher than in the control group (0.27±0.06) (P<0.05) (Fig 5J). As the experiment continued for 72 h, the relative number of surviving HUVECs remained higher in the DP-CM group (0.23±0.03) than in the DMEM group (0.12±0.02) (P<0.05). To examine the effect of DP-CM on anti-apoptosis induced by serum starvation and hypoxia, after 48 h TUNEL assay was performed (Fig 5A-H). DP-CM reduced apoptosis of HUVECs from 27.8±6% to 14.6±4% (P<0.001) (Fig 5I).

To identify mechanisms of cytoprotection of DP-CM against ischemia/reperfusion, H_2O_2-induced cell death assay was performed. After H_2O_2 exposure, 12.8% of the cells (Annexin V+/7-AAD-) were in the early apoptotic stage, which was significantly reduced to 5.16% in the DP-CM treated HUVECs, similar to 2.39% for the negative control (Fig 6). The number of double-positive cells in the late stage of apoptosis (Annexin V+/7-AAD+) was also significantly decreased in the DP-CM treated cells, from13.5% to 1.84% (Fig 6). The unlabeled cells were markedly increased in the DP-CM-treated cells, from 72% to 89.9% (Fig 6). Results demonstrated that DP-CM significantly protected against H_2O_2-induced apoptosis of HUVECs.

3.5 DP-CM was Shown to Significantly Improve the Recovery of Persistent Blood Flow in Ischemic Hindlimb

Blood perfusion in the ischemic hindlimb was measured 24 days after localized intramuscular administration of DP-CM or serum-free DMEM (Fig 7A). The LDPI index showed that the mice treated with DP-CM had a marked improvement in blood perfusion in the ischemic limb (77%±11%). In contrast, in the mice receiving DMEM showed blood perfusion rates around (45%±6%) (Fig 7B). This suggests that the effects of local intramuscular DP-CM injection enhanced neovascularization of the ischemic hindlimb.

3.6 DP-CM Increase the Density of CD31-positive Microvessels

Immunohistochemical analysis was performed in order to evaluate neovascularization levels throughout the DP-CM

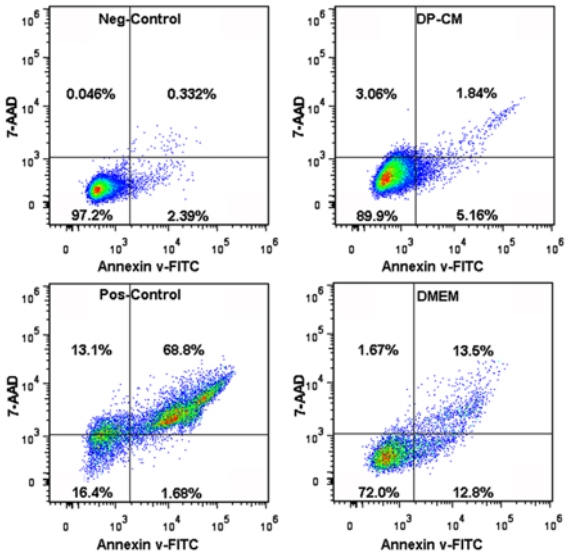

Figure 6. Effects of DP-CM on apoptotic HUVECs induced with H_2O_2. HUVECs were treated by H_2O_2 (500 μM) and incubated with either DP-CM or DMEM for 4 h and stained with Annexin V-FITC and 7-AAD. The distribution of apoptotic cells was analyzed using Flow cytometer. Only 7-AAD positive cells are dead. Cells showing Annexin V and 7-AAD double positive represent the stage of late apoptosis. Live cells were not labeled with Annexin V and positive, whereas Annexin V-labeled cells represent the early stage of apoptosis. Positive control, HUVECs treated with 10 mM H_2O_2. Negative control, HUVECs incubated with DMEM.

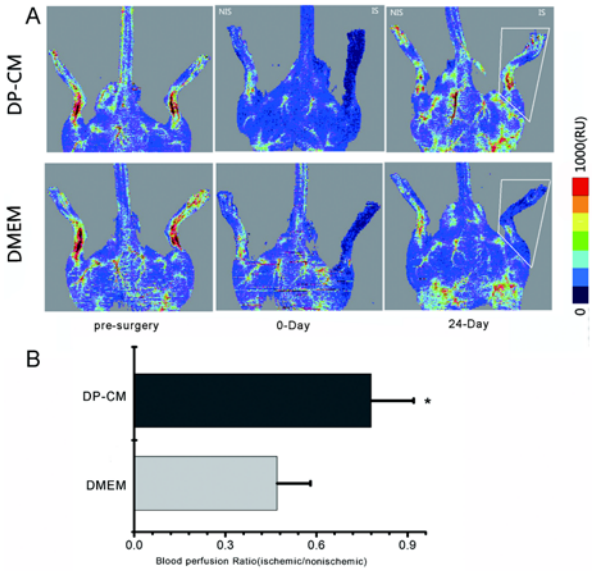

Figure 7. (A) Representative images of laser Doppler blood perfusion. The ischemic hindlimb blood perfusion increased markedly after 24 days in the DP-CM treated group. (B) Quantitative analysis of hindlimb blood perfusion using the LDPI index, the ratio of ischemic to nonischemic hindlimb blood perfusion. The increase in the LDPI index was significant in the DP-CM treated group compared to the DMEM-treated group. Data presented are mean±SEM.

treatment. CD31-positive microvessels were counted in the ischemic hindlimb muscle tissue of mice (Fig 8A). The number of CD31-positive microvessels (6.2±1.1)/HPF was markedly higher in the DP-CM group than in the control group (2.3±0.3)/HPF (Fig 8B). Results indicated that DP-CM administration could increase the number of CD31-positive microvessels that participated in the formation of functional vascular structures.

3.7 Profiles of Angiogenic Factors in DP-CM

The DPSCs and AD-MSCs secrete a variety of growth factors and cytokines into their medium in the normal culture. Concentrations of 14 growth factors were measured using ELISA and liquidchip assay. Results indicated that DSPCs secreted relatively higher levels of SDF-1(504±173), TGF-β (3555±927), MCP-1(8240±417), FGF-2 (138.9±10), PDGF-BB (122±7.9) and VEGF (6882±1644) compared with AD-MSCs and fibroblast ($p<0.01$) (Table 1). However, AD-MSCs showed a special profile of cytokines with immunoregulation potential such as IL-10 (788±46) and IL-8 (1194±133) (Table 1). And the results showed that the DSPCs showed no significant differences in their secretion level of such cytokines as EGF

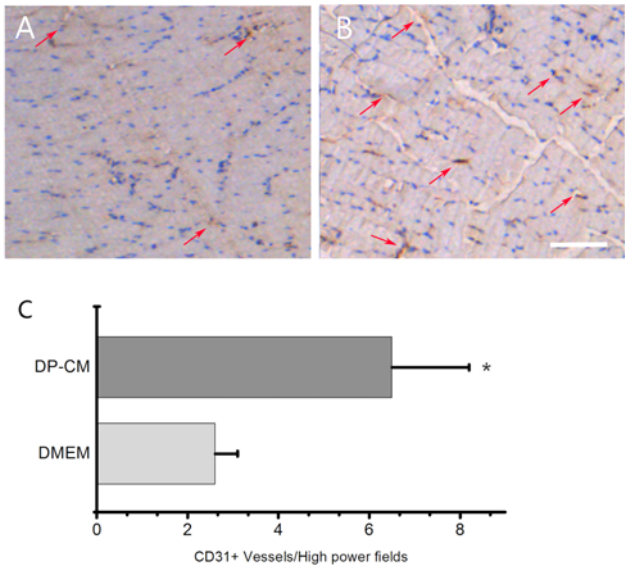

Figure 8. (A) Representative samples of CD31 positive microvessels in ischemic hindlimb muscles. (B) Quantitative analysis of CD31-positive microvessels density in ischemic hindlimb muscle tissue (n=6). (C) The DP-CM treatment group showed significant increase in the number of CD31-positive microvessels, compared with the control. Red arrows indicate CD31 positive microvessels. *$p < 0.05$ relative to the respective DMEM group. Scale bar in image =100 μm.

Table 1. Levels of cytokines and growth factors in DP-CM and conditioned medium derived from AD-MSCs, fibroblast. After 80% confluence, cells were changed with DMEM last for 3 days. Measurements of cytokines and growth factors were performed by ELISA and liquidchip assays. The data shown are the mean±S.D. (n = 3).

Name	Assay	DPSCs (pg/ml)	AD-MSCs (pg/ml)	Fibroblast (pg/ml)
IGF-1	ELISA	820±114	1035.4±207	27±11
VEGF	ELISA	6882±1644	340±117	340±117
bFGF	ELISA	269±180	ND	315±90
SDF-1	ELISA	504±173	43±7	74±8
MCP-1	ELISA	8240±417	97.3±21	8.1±2
TGF-β	ELISA	3555±927	222.95±25	1605±335
EGF	LC	<3.6	<3.6	<3.6
FGF-2	LC	138.9±10	15.7±5	28.9±9
TGF-α	LC	ND	ND	ND
IL-10	LC	ND	788±46	1.66±0.6
PDGF-BB	LC	122±7.9	<2.45	29.1±12
IL-8	LC	415±126	1194±133	285±172
IP-10	LC	135±19.8	<125.9±11	36.4±15
MCP-1	LC	11388±1917	4248±1225	914±213
VEGF	LC	2121.3±621	230.3±13	144±33
MIP-1a	LC	ND	<1.46	8.14±4

Comparison of the levels of cytokines and growth factors. Concentrations are expressed the means ± SD of three independent experiments. LC, liquidchip assay. ND, not detectable.

and IP-10 compared with the AD-MSCs (Table 1).

4. Discussion

In the present study, the ability of DP-CM to promote angiogenesis and blood perfusion was investigated in a mouse model of hindlimb ischemia. First, results showed the DPSCs to resemble MSCs with respect to multipotency (Fig 1). Second, the formation of HUVECs tube formation assays and migration assay indicated that DP-CM could stimulate angiogenesis and accelerated the migration of VSMCs and HUVECs (Fig 4). This effect appears to be specific to neovascularization, as indicated by the fact that DP-CM contains angiogenic growth factors, which foster the endothelization. Third, Hypoxia and reperfusion induced apoptosis is a major cause of ischemia injury. Results indicated that DP-CM significantly protected HUVECs from loss of viability relative to the DMCM group (Fig 5,6).

Furthermore, we applied the DP-CM to the mouse model of hindlimb ischemia, we found that local infusion of DP-CM significantly increased blood perfusion and CD31-positive microvessels density in ischemic areas (Fig 7,8). Since the blood flow to the ischemic limbs is almost completely blocked, DP-CM was injected locally rather than intravenously. For this reason, more cytokines accumulated at the site of the ischemic tissue, DP-CM was found to confer neovascularization *in vitro* and *in vivo*.

Hindlimb ischemia is characterized by a decrease in blood flow, subsequent blood vessel loss and tissue damage. The objective of therapeutic angiogenesis is to improve blood perfusion in the ischemic region through new vessel formation and reduce hypoxia/oxidative stress caused apoptosis. MSCs have been utilized for treatment of ischemic diseases through paracrine mechanisms. In clinical setting, stem cells are usually injected into tissue, where the excess inflammation and hypoxic conditions may lead to poor survival of transplanted cells.[25] Several previous reports have suggested that the cytoprotective effects and angiogenesis ability of stem cells may be due to the secretion of specific cytokines from the stem cells. For instance, SDF-1derived from cardiac stem cells mediated cardioprotection following acute I/R injury through recruitment of stem cells and promote cell survival.[26] In addition, a few reports have demonstrated that bone marrow-derived mesenchymal stem cell conditioned media (MSC-CM) led to angiogenesis and regeneration in myocardial infarction and in calvarial bone defects.[27,28] In this study, although the mechanism underlying the therapeutic effects of the DP-CM is unclear, though it does appear to be complicated. The paracrine mechanisms associated with MSCs may play an important role in the angiogenic process.[25,29]

Angiogenesis is a complex process involving various growth factors, chemokines, and mural cells, all of which play different roles in promoting and refining this process. Although the aim of this study was not the identification which factor responsible for the angiogenic properties of DP-CM, we have confirmed that DPSCs release relative high amount angiogenic factors and cytokines such as MCP-1, bFGF, IGF-1, TGF-β, PDGF-BB, SDF-1 and VEGF in medium compared with AD-MSCs or fibroblast. According to previous reports, VEGF can promote endothelial cell proliferation, migration, and tube-forming activity.[30,31] PDGF induces stabilization of the newly formed blood vessels by recruiting progenitor cells to the nascent vasculature.[32] IGF-1 induces resident cardiac stem cell migration, proliferation and differentiation, resulting in cardiac regeneration.[33] SDF-1 was found to be associated with improved survival and engraftment in an infarcted hearts and with stem cell recruitment.[34-36] Cytokines have not only individual effects, but one cytokine may potentiate the effect of another.

MCP-1 mediates TGF-β, which promotes angiogenesis by stimulating VSMCs migration.[37]

Accordingly, the blend of growth factors of DP-CM disclosed strong capacity of angiogenesis, anti-apoptosis, and migration. In this work, such growth factors as MCP-1, SDF-1 and TGF-β of DP-CM may stimulated the migration of VSMCs and HUVECs and the properties of facilitated HUVEC tube formation treated with DP-CM may be due to the angiogenic factors IGF-1, VEGF and PDGF-BB secreted from the DPSCs. DP-CM treatment group showed the reduction in the number of TUNEL positive cells under hypoxic condition indicating that anti-apoptotic factors of DP-CM has some useful effects for the recovery of damaged cells. The DP-CM treated cells showed a significantly decreased percentage of H_2O_2 induced early and late apoptotic cells compared with the control group (Fig 6). This enhanced viability of HUVECs may be also due to the anti-apoptotic factors of DP-CM, such as, SDF-1, VEGF and IGF-1.[38-40]

The most obvious limitation of protein-based therapy is the necessity to maintain concentration with a long-lasting therapeutic effect. The presented data therefore suggest that two injections of concentrated DP-CM leads to a substantial increase in blood flow in the ischemic hindlimb and the formation of a persistent capillary network as detected 24 days after injection. In conclusion, DP-CM treatment was found to enhance blood perfusion and vascular remodeling in a mouse model of hindlimb ischemia, suggesting that a DP-CM based therapeutic agent may be valuable in clinical settings.

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Conflict of Interest: ChongYang Shen, Lin Li, Ting Feng, JinRong LI, MeiXing Yu, Qiao Lu, and Hong Li declare that they have no conflict of interest.

Ethical Statement: Human dental pulp samples were collected from donors with their consent at the Sichuan University Hospital of Stomatology.

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