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Effects of Conditioned Medium of Adipose-Derived Stem Cells Exposed to Platelet-Rich Plasma on the Expression of Endothelial Nitric Oxide Synthase and Angiogenesis by Endothelial Cells

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Abstract: Platelet-rich plasma (PRP) and adipose-derived stem cells (ADSCs) are known to secrete angiogenic factors that contribute to the treatment of intractable ulcers. The combination of PRP and ADSCs may enhance their angiogenic effects. However, it remains unclear whether treatment of ADSCs with PRP influences angiogenesis. We studied whether the conditioned medium from PRP-treated ADSCs under hypoxic conditions exerts angiogenic effects. Although PRP stimulated the proliferation of ADSCs obtained from rats, it decreased the mRNA levels of vascular endothelial growth factor, hepatocyte growth factor, and TGF-β1, but not of basic fibroblast growth factor, under hypoxia. The conditioned medium of PRP-treated ADSCs inhibited endothelial nitric oxide synthase phosphorylation, decreased NO production, and suppressed tube formation in human umbilical vein endothelial cells. Transplantation of ADSCs alone increased both blood flow and capillary density of the ischemic limb; however, its combination with PRP did not further improve blood flow or capillary density. This suggests that both conditioned medium of ADSCs treated with PRP and combination of PRP with ADSCs transplantation may attenuate the phosphorylation of endothelial nitric oxide synthase and angiogenesis.

Key Words: platelet-rich plasma, adipose tissue-derived stem cells, eNOS, angiogenesis

(*Ann Plast Surg* 2023;90: 171–179)

The improvement in blood flow in tissues can facilitate wound healing.[1] Although treatments to improve blood flow are available,[2,3] intractable ulcers (which do not respond to any treatment) pose a clinical problem. Thus, alternative methods for improving blood flow are being sought for treating intractable ulcers.

Bone marrow stem cells (BMCs) secrete angiogenic factors, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and basic fibroblast growth factor (bFGF).[4] They have been applied to treat peripheral artery disease (PAD) and pressure ulcers.[5] As a limited number of BMCs can be collected from the bone marrow, alternative cell sources are required. Adipose tissue-derived stem cells (ADSCs) are known to promote angiogenesis,[6] and their collection is less invasive than that of BMCs.[7,8] Adipose-derived stem cells have been clinically applied to treat ischemic limb diseases and to reconstruct breast tissue. We have previously shown that ADSCs secrete angiogenic factors, such as VEGF, HGF, and bFGF, and they suppress ischemia in rat models of limb ischemia or myocardial infarction.[9,10] Vascular endothelial growth factor activates the phosphatidyl-inositol-3 kinase/protein kinase B (Akt) pathway and increases the production of nitric oxide (NO) by vascular endothelial cells, leading to improved endothelial function and angiogenesis.[11] A reduction in NO production through impaired VEGF signaling in patients with diabetes has been reported to cause the underlying development and refractoriness of diabetic skin ulcers.[12]

Platelet-rich plasma (PRP) is a plasma component extracted by aggregating platelets, which secrete anti-inflammatory cytokines and release angiogenic factors such as VEGF and HGF. Platelet-rich plasma and ADSCs are used to treat intractable ulcers[13] because they promote angiogenesis and tissue regeneration, respectively.[14,15] While PRP and ADSCs individually improve intractable skin ulcers and critical limb ischemia,[16] their combination yields better results.[17] Therefore, the combination of PRP and ADSCs is expected to enhance their angiogenic effects. The proliferation of ADSCs was reported to be significantly impaired by PRP,[18,19] which suggests that PRP, at a certain level, might influence the angiogenic effect of ADSCs. It remains unknown whether conditioned medium from PRP-treated ADSCs influences the eNOS/NO-dependent angiogenesis of endothelial cells. In this study, we investigated whether a conditioned medium from rat PRP-treated ADSCs under hypoxic conditions can have angiogenic effects on human umbilical vein endothelial cells (HUVECs), and whether the combination of PRP with ADSCs transplantation in rats would show angiogenic effects in a BALB/c nu/nu mouse ischemic limb model. Contrary to our hypothesis that the combination of PRP with ADSC transplantation would enhance the angiogenic action of ADSCs to improve endothelial function, in the present study, we found that pretreatment with PRP might abolish the angiogenic action of ADSCs in vitro and in vivo.

MATERIALS AND METHODS

Animal Care

Seven-week-old male Lewis rats weighing 200 to 250 g were purchased from Japan SLC, Inc (Shizuoka, Japan) and 7-week-old male Balb/c-nu/nu mice were purchased from SHIMIZU Laboratory Supplies Co, Ltd (Kyoto, Japan). The Institutional Animal Care and Use Committee of Tottori University approved all the experimental protocols (18-Y-7, 21-Y-3).

Received July 19, 2022, and accepted for publication, after revision October 26, 2022.
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Conflicts of interest and sources of funding: None declared.
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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.annalsplasticsurgery.com).

ISSN: 0148-7043/23/9002–0171
DOI: 10.1097/SAP.0000000000003368

TABLE 1. Primers Used for Reverse Transcription Polymerase Chain Reaction

Gene Symbol	Forward Primer	Reverse Primer	Probe No.
VEGF-A	5′-TTAAACGAACGTACTTGCAGATG-3′	5′-TCTAGTTCCCGAAACCCTGA-3′	#4
bFGF	5′-TCTTCCTGCGCATCCATC-3′	5′-GCTTGGAGCTGTAGTTTGACG-3′	#7
TGFβ1	5′-TCAGACATTCGGGAAGCAGT-3′	5′-ACGCCAGGAATTGTTGCTAT-3′	#56
HGF	5′-GATTGGATCAGGACCTTGTGA-3′	5′-CCATTCTCATTTTGTGTTGTTCA-3′	#49
β-Actin	5′-CTAAGGCCAACCGTGAAAAG-3′	5′-GCCTGGATGGCTACGTACA-3′	#115

Extraction of Activated PRP

Platelet-rich plasma was extracted from blood as previously described.[20] In this study, we used rat blood for PRP extraction. Peripheral blood (5–10 mL) collected in the presence of EDTA (15 mg) was centrifuged at 450*g* for 7 minutes, and plasma with buffy coat was collected and centrifuged at 1600*g* for 5 minutes. Platelets that accumulated in the pellet were used as PRP components. Blood (1 mL) was kept standing for 30 minutes at room temperature without anticoagulant and was then centrifuged at 2000*g* for 5 minutes. The supernatant was collected as the autologous thrombin. A mixture of 0.5 M $CaCl_2$ and autologous thrombin 1:1 (vol/vol) was prepared in advance as the activator. A mixture of PRP and activator at 10:1 (vol/vol) was incubated for 10 minutes at room temperature. Activated PRP was centrifuged at 90*g* and then at 9000*g* for 10 minutes and stored at −80°C until further use.

Cell Culture Under Normoxic and Hypoxic Conditions

Freshly isolated ADSCs were obtained from the inguinal fat pads of rats. Adipose tissue was minced and digested with 1 mg/mL type I collagenase (Wako, Osaka, Japan) at 37°C for 1 hour. After filtration through a 100-μm strainer to remove adipocytes, ADSCs were harvested by centrifugation at 620*g* for 5 minutes. The cells were incubated in DMEM supplemented with 20% FBS and 1% penicillin-streptomycin-L-glutamine. The cells were cultured for 2 to 5 days until they reached confluency. These cells were defined as passage "0" cells. For all experiments, we used cells at passage "3." Hypoxic conditions were created using an AnaeroPack system (Mitsubishi Gas Chemical, Inc, Tokyo, Japan). Adipose-derived stem cells were cultured at 37°C under normal (21% O_2) or hypoxic (<2% O_2) conditions for 24 hours in the absence or presence of PRP at the indicated concentrations.

Adipose-Derived Stem Cell Proliferation Assay

Adipose-derived stem cells at passage 3 were seeded at a density of 1.5×10^5 cells/mL in 6-well plates and cultured overnight in DMEM supplemented with 20% FBS and 1% penicillin-streptomycin-L-glutamine. Subsequently, ADSCs were cultured with 3 different types of media (serum-free DMEM only, 5% PRP + serum-free DMEM, and 10% PRP + serum free DMEM) overnight. The number of total and living cells was determined using a TC20 Automated Cell Counter (Bio-Rad, Calif) according to the manufacturer's instructions.

Real-Time Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA was extracted from ADSCs using an RNeasy Mini Kit (Qiagen, Inc, Valencia, Calif). Real-time reverse transcription polymerase chain reaction analysis of VEGF, HGF, TGF-β1, bFGF, and β-actin was performed using ViiA7 (Applied Biosystems, Foster City, Calif). The respective mRNA levels were expressed relative to β-actin levels. mRNA levels were determined using the comparative C_t method and expressed as $2^{-\Delta Ct}$. The primers used are listed in Table 1.

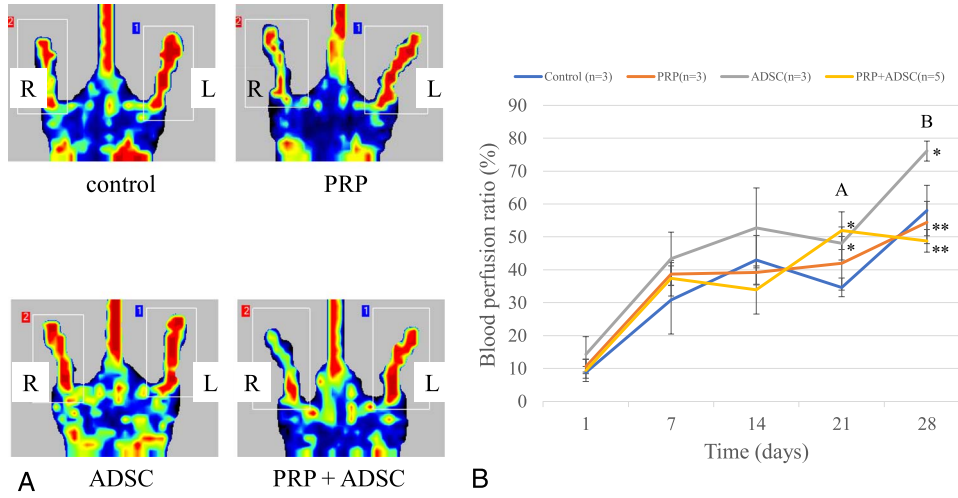

FIGURE 1. Effects of injecting PRP and ADSCs in combination on blood flow in the ischemic limb. A, Representative images of ischemic lower limbs of Balb c-nu/nu mice in control (upper left panel: control) and rat PRP injection groups (upper right panel: PRP), rat ADSC transplanted group (lower left panel: ADSC), and rat ADSC transplanted with rat PRP injection group (lower right panel: PRP + ADSC). The images were obtained on day 28 after ligation of the right external iliac artery. B, Average time-dependent recovery of blood perfusion in ischemic hind limbs of Balb c-nu/nu mice. Each bar represents the mean ± S.D. Control group: given saline (n = 3); PRP group: PRP injection group (n = 3); ADSC group: ADSC transplanted group (n = 3); and PRP + ADSC group: ADSC transplanted with PRP injection group (n = 5). **P* < 0.05 versus control group, B: **P* < 0.05 or ***P* < 0.01 versus ADSC group.

Conditioned Medium of ADSCs Treated With PRP

Adipose-derived stem cells (3.0×10^5 cells/6-well plate) were cultured overnight. The medium was then replaced with one of the following three media: serum-free DMEM, 5% PRP + serum-free DMEM, or 10% PRP + serum-free DMEM. The cells were cultured at 37°C under normoxic (21% O_2) or hypoxic (<2% O_2) conditions for 24 hours in the absence or presence of PRP. Each conditioned medium was collected and stored at −80°C until use.

Western Blot Analysis

Human umbilical vein endothelial cells (Takara Bio Shiga, Japan) were cultured in conditioned medium of ADSCs treated with 0%, 5%, or 10% PRP under normoxic or hypoxic conditions. The cells were collected and lysed in RIPA buffer. After the removal of insoluble materials, protein concentrations were determined using a Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Calif). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene fluoride membranes. The membranes were probed with primary antibodies against eNOS (BD Transduction Laboratories, Franklin Lakes, NJ), phosphorylated eNOS (p-eNOS, Ser1177), phosphorylated VEGF receptor 2 (Tyr1175; Cell Signaling Technology, Danvers), VEGFA (VG-1, Abcam Cambridge, United Kingdom), and β-actin. Blots were developed using Pierce ECL Plus western blotting substrate (Thermo Fisher Scientific, Mass). The intensities of the bands were quantified using the ImageJ software (NIH). In some experiments, ADSCs were also used for western blot analyses.

Measurement of NO Production

Nitric oxide concentration was determined using a colorimetric kit (QuangiChrom D2NO-100 Nitric Oxide Assay Kit; BioAssay Systems, Hayward, Calif). Nitric oxide concentrations in the culture medium of HUVECs treated with conditioned ADSC medium exposed to various PRP concentrations were determined by measuring absorbance at 540 nm using a microplate reader (Sunrise Rainbow RC-R; Tecan Japan Co, Ltd, Kanagawa, Japan). Nitric oxide concentration was normalized to the number of HUVECs.

Tube Formation Assay

Matrigel was thawed, suspended in 96-well plates, and allowed to solidify at 37°C for 30 minutes. HUVECs were seeded at a density of 3.5×10^4 cells per well and cultured in medium (serum-free DMEM) containing different concentrations of PRP (0%, 5%, and 10% PRP) for 12 to 14 hours. Tubular structures were examined under a microscope. Quantification was performed by measuring the number of branch points.

Preparation of a Unilateral Hind Limb Ischemia Model

Unilateral limb ischemia was induced in Balb-6 nu/nu mice (males, 7-weeks-old) as described previously.[21] On day 0, mice were

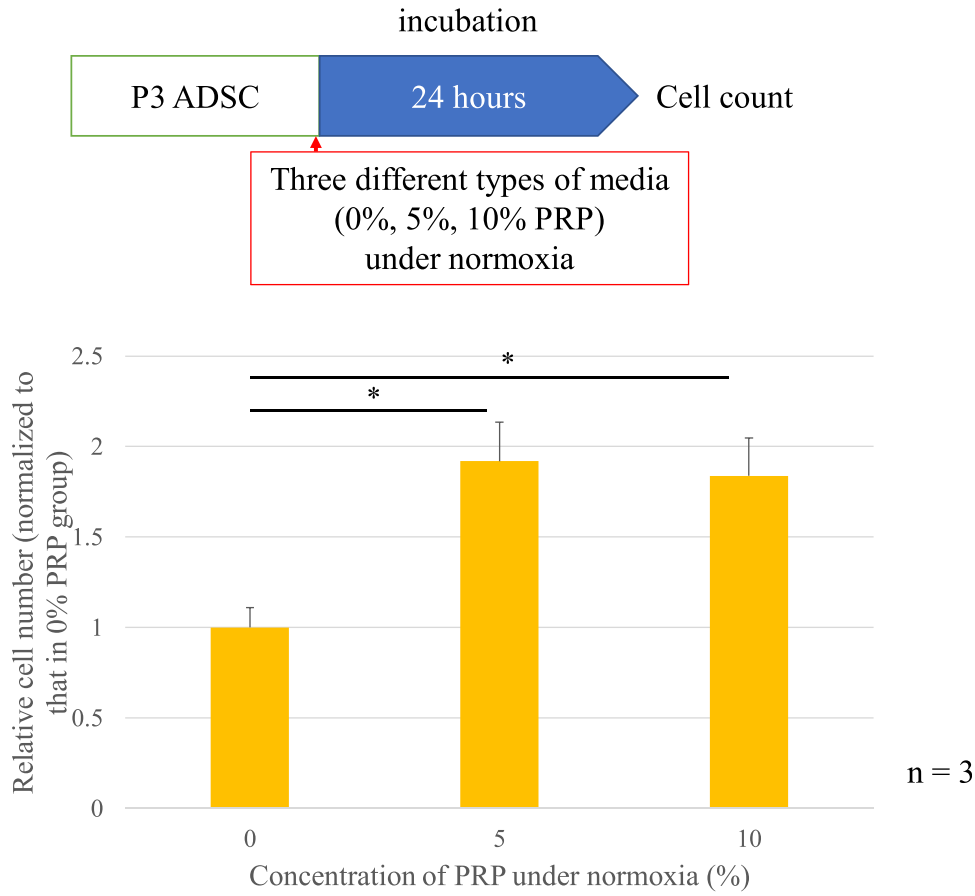

FIGURE 2. Effects of PRP on the proliferation of ADSCs. The schematic shows the experimental protocol and the graph shows the comparison of the relative cell numbers under different treatment conditions. The cell numbers were normalized to that in 0% PRP conditions. There was a significant increase in the number of cells in the 5% and 10% PRP groups compared with that in the 0% PRP group (n = 3). All measurements were performed after 24 hours of culture. *$P < 0.05$ versus 0% PRP.

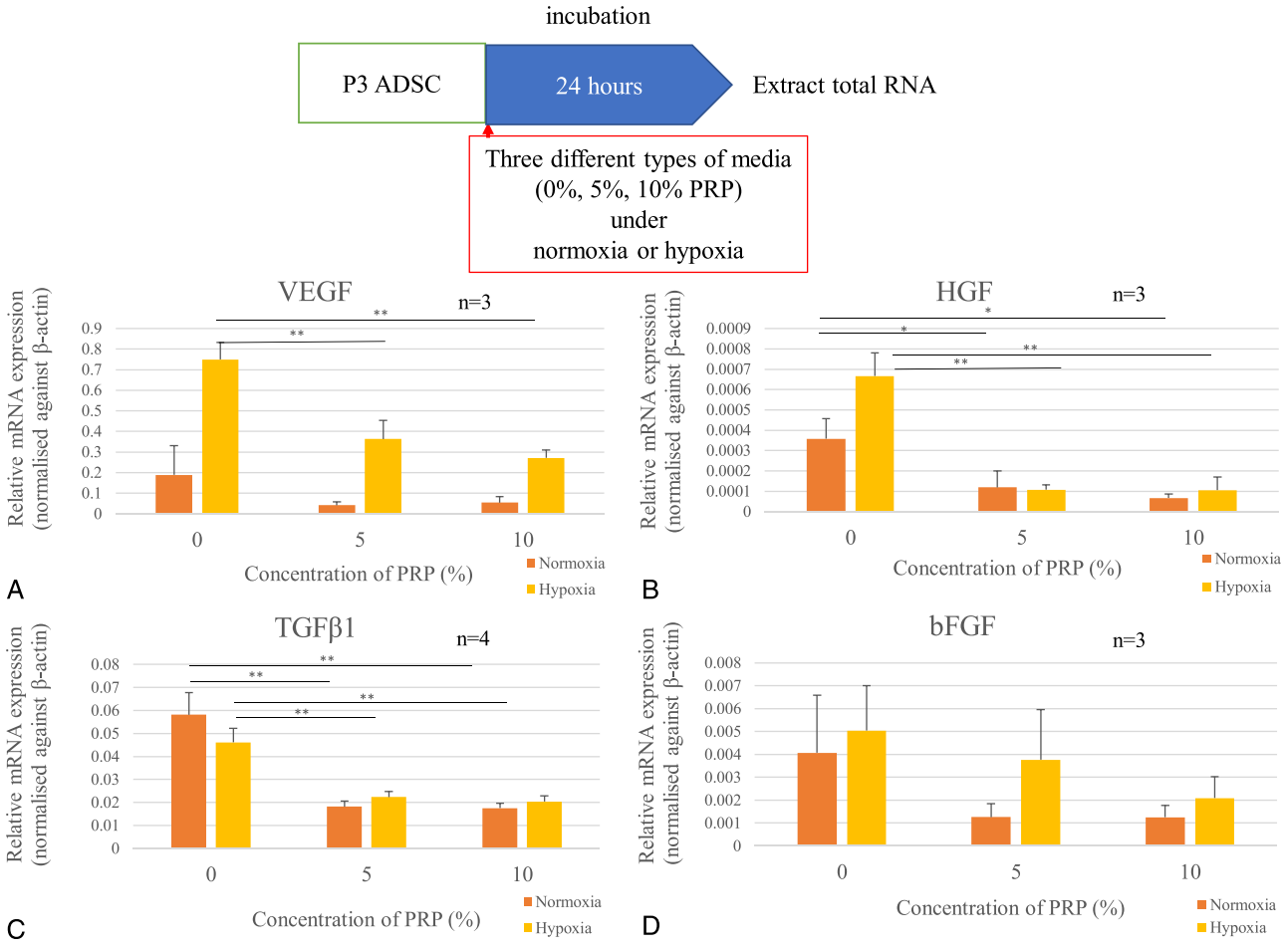

FIGURE 3. Effect of PRP on the mRNA expression of angiogenic factors of ADSCs under normoxic and hypoxic conditions. The schematic shows the experimental protocol and graphs show the relative mRNA expression levels of VEGF (A), HGF (B), TGF-β1 (C), and bFGF (D) in ADSCs cultured in medium treated with 0%, 5%, or 10% PRP under normoxia or hypoxia. mRNA expression levels were normalized to that of β-actin. All the measurements were made after 24 hours of culture. $*P < 0.05$, $**P < 0.01$ versus 0% PRP, n = 3–4.

anesthetized by intraperitoneal injection of xylazine hydrochloride (5 mg/kg; Bayer, Tokyo, Japan) and ketamine hydrochloride (80 mg/kg; Sankyo Pharmaceuticals, Tokyo, Japan), and the proximal right external iliac artery was ligated using 8-0 silk (BEAR Medic, Chiba, Japan). Rat-derived ADSCs and/or rat-derived PRP were transplanted into a unilateral ischemic hind limb model of BALB/c nu/nu mice. Samples (10 μL) from each group were injected at 4 different points in the mouse thigh adductor muscle. Mice in the control, PRP, ADSC, and PRP and ADSCs groups were injected with 40 μL PBS, 40 μL PRP, 1.0×10^6 ADSCs suspended in 40 μL PBS, and 1.0×10^6 ADSCs suspended in 40 μL PRP, respectively.

Laser Doppler Blood Flow Analysis

At 1, 7, 14, 21, and 28 days after surgery, lower limb blood flow was measured using a laser Doppler blood flow (LDBF) analyzer (Permed, Stockholm, Sweden). As shown in Figure 1, the Doppler signal was used as an index of microvascular perfusion in the muscle area depicted by the cursor. We measured LDBF 3 times at 1-minute intervals, and the values were averaged. The results are expressed as the ratio of blood flow in the untreated limb.

Measurement of Capillary Density

On day 28, the adductor skeletal muscle was isolated from the ischemic limb, embedded in Tissue-Tek 4593 optimal cutting temperature compound (OCT; Sakura Finetek Japan, Tokyo, Japan), and snap-frozen in liquid nitrogen without sucrose treatment. We used unfixed samples to avoid any antigenic changes due to fixation. Cryostat sections (8 μm thick) of each specimen were prepared. The sections were incubated with antibodies specific to anti-CD31 antibody (BD Biosciences, San Diego, Calif) that were labeled with biotin and detected by avidin-biotin complex (Vector Laboratories, Burlingame, Calif) for 2 hours. Labeled cells were defined as vascular endothelial cells. Capillary density was expressed as the number of anti-CD31 antibody-positive features per skeletal muscle fiber. Five randomly selected fields from transverse sections were analyzed per animal.[21]

Data Analysis

All data are expressed as mean ± standard error of the mean (SEM). Student *t* test and analysis of variance with Tukey post hoc test were used for comparisons between 2 groups and multiple (3 or more)

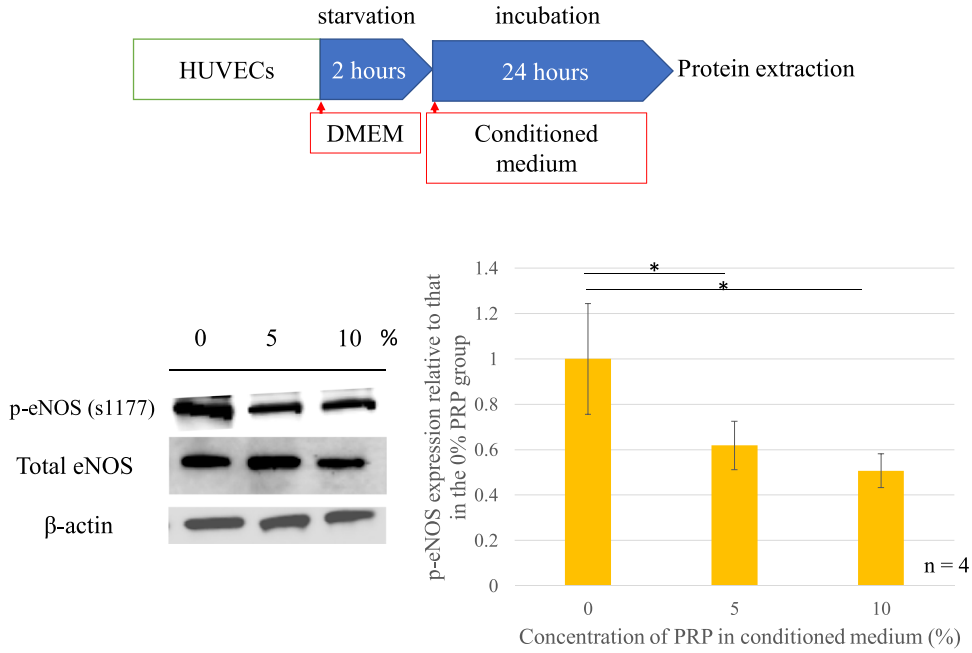

FIGURE 4. Effects of conditioned medium of PRP-treated ADSCs on eNOS phosphorylation in HUVECs under hypoxia. The schematic shows the experimental protocol; the lower left panel shows the total and phosphorylated eNOS levels in HUVECs exposed to the conditioned medium of 5% and 10% PRP-treated ADSCs under hypoxic conditions compared with that in cells exposed to the conditioned medium alone (0%), and the lower right panel shows the summary of the effects of conditioned medium of PRP-treated ADSCs on eNOS phosphorylation in HUVECs under hypoxia (n = 4). The relative expression level of eNOS was normalized to that in the 0% PRP group. All measurements were made after 24 h of culture. *$P < 0.05$ versus 0% PRP.

groups, respectively. Statistical significance was set at a P value less than 0.05.

RESULTS

Effects of PRP on the Proliferation of ADSCs

We evaluated the proliferation of ADSCs treated with 0%, 5%, or 10% PRP (Fig. 2). Data obtained from 3 experiments showed significant stimulatory effects of 5% and 10% PRP compared with that of 0% PRP ($P < 0.05$). There was no significant difference between the 5% and 10% PRP groups.

Effects of PRP on mRNA Expression of Angiogenic Factors in ADSCs Under Normoxic and Hypoxic Conditions

We determined the mRNA levels of VEGF, HGF, TGF-β1, and bFGF in ADSCs treated with either 5% or 10% PRP under normoxia and hypoxia (Fig. 3). The mRNA levels of VEGF and HGF, but not of TGF-β1 and bFGF, increased under hypoxia compared with those under normoxia. Both 5% and 10% PRP significantly suppressed the mRNA levels of VEGF, HGF, and TGF-β1, but not of bFGF, under hypoxia.

Effects of Conditioned Medium of PRP-Treated ADSCs on eNOS Phosphorylation in HUVECs Under Hypoxia

eNOS expression regulates vascular endothelial cell angiogenesis. We evaluated the protein levels of total and phosphorylated eNOS in HUVECs exposed to the conditioned medium of ADSCs treated with 5% or 10% PRP under hypoxia. Figure 4 lower left panel shows the total protein expression of eNOS and phosphorylated eNOS in HUVECs exposed to the conditioned medium of ADSCs treated with 0%, 5%, and 10% PRP under hypoxia. Phosphorylation of eNOS was remarkably suppressed in HUVECs exposed to the conditioned medium of 5% and 10% PRP-treated ADSCs under hypoxic conditions compared with that in HUVECs exposed to the conditioned medium of 0% PRP-treated ADSCs. Data obtained from 4 experiments indicated that conditioned medium of 5% and 10% PRP-treated ADSCs under hypoxic conditions suppressed the phosphorylation of eNOS ($P < 0.05$; Fig. 4, lower right panel).

Effects of the Conditioned Medium of PRP-Treated ADSCs Under Hypoxia on NO Production by HUVECs

We examined NO production in HUVECs exposed to conditioned medium of ADSCs treated with PRP at the indicated concentrations (Fig. 5). Data obtained from 4 experiments showed that the conditioned media of 5% and 10% PRP-pretreated ADSCs under hypoxia significantly suppressed NO production by HUVECs.

Effects of the Conditioned Medium of PRP-Pretreated ADSCs on Tube Formation by HUVECs

We examined whether the conditioned medium of PRP-treated ADSCs could influence tube formation by HUVECs. Tube formation by HUVECs was observed when they were exposed to conditioned medium of ADSCs with 0% PRP under hypoxia (Fig. 6). The conditioned medium of ADSCs treated with 5% and 10% PRP under hypoxia suppressed tube formation in HUVECs (Fig. 6A). Data obtained from 4 experiments indicated significant effects of the conditioned medium of ADSCs treated with 5% and 10% PRP (Fig. 6B).

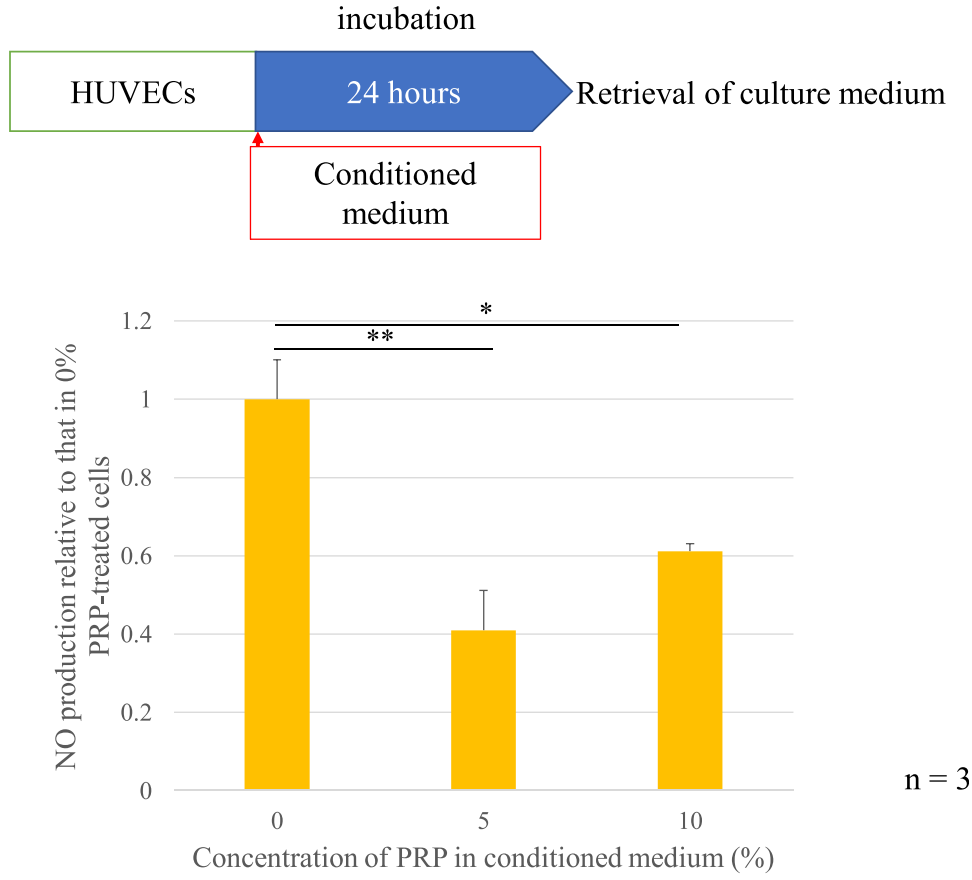

FIGURE 5. Effects of the conditioned medium of PRP-treated ADSCs under hypoxia on NO production in HUVECs. The schematic shows experimental protocol and the graph shows the relative NO production in HUVECs cultured with ADSC culture medium treated with 5% and 10% PRP under hypoxic conditions normalized to that in the 0% PRP group (n = 3). Nitric oxide production in HUVECs was normalized to the cell number. All the measurements were made after 24 hours of culture. *$P < 0.05$, **$P < 0.01$ versus 0% PRP.

Effects of PRP Injection on Blood Flow and Capillary Density in the Ischemic Limb of Mouse Transplanted With ADSCs

To evaluate the angiogenic effect of ADSCs, we analyzed LDBF in the control group (n = 3), rat PRP injection group (n = 3), rat ADSC-transplanted group (n = 3), and rat ADSC-transplanted with rat PRP injection group (n = 5).

Figure 1A shows the representative LDBF of the ischemic limb (right side) of BALB/c nu/nu mice in each group on day 28. Laser Doppler blood flow in ADSC-transplanted mice was higher than that in the control, PRP injection, and ADSC-transplanted with PRP injection groups. Figure 1B shows the summary data on the time-dependent recovery of blood flow after surgery (day 1) up to day 28, obtained in the LDBF experiments. The perfusion recovery in the ADSC-transplanted group was significantly higher on day 28 after ischemia than that in the control group and ADSC-transplanted with PRP injection group. As shown in Figure 7, measurement of capillary density using immunostaining of tissue sections prepared from the thigh adductor muscle of Balb c-nu/nu mice on day 28 with anti-CD31 antibody confirmed that the vascular density was higher in the ADSC transplanted group than in the control and ADSC transplanted with PRP injection groups.

DISCUSSION

In this study, we demonstrated that PRP[1] stimulated the proliferation of ADSCs[2]; PRP inhibited the expression of VEGF, HGF, and TGF-β1 mRNAs in ADSCs under hypoxic conditions[3]; conditioned medium from PRP-treated ADSCs suppressed phosphorylation and NO production of eNOS in HUVECs[4]; conditioned medium from PRP-treated ADSCs suppressed tube formation in HUVECs[5] (Supplemental Fig. 3, http://links.lww.com/SAP/A796); and the transplanted ADSCs with PRP injection did not improve blood flow or capillary density of the ischemic limb.

Platelet-rich plasma stimulates the proliferation of ADSCs in a dose-dependent manner, as expected from the presence of platelet-derived growth factor–BB in PRP via the ERK1/2, PI3K/Akt, and JNK signaling pathways.[19] In the present study, we also found that PRP stimulated the proliferation of ADSCs, which is consistent with the findings of Hara et al,[20] whereas the proliferation of ADSCs stimulated by PRP was not concentration-dependent. Platelet-rich plasma did not influence ADSC death, suggesting that the proliferation of ADSCs stimulated by PRP might be saturated.

Platelet-rich plasma contains α-granules of platelets and several cytokines, including platelet-derived growth factor, TGF-β, VEGF, IGF, EGF, and bFGF.[22–24] Samberg et al[25] reported that human PRP increased the mRNA expression levels of VEGF-A and ANGPT-1 in cultured human ADSCs but decreased the mRNA levels of ANGPT-2. In

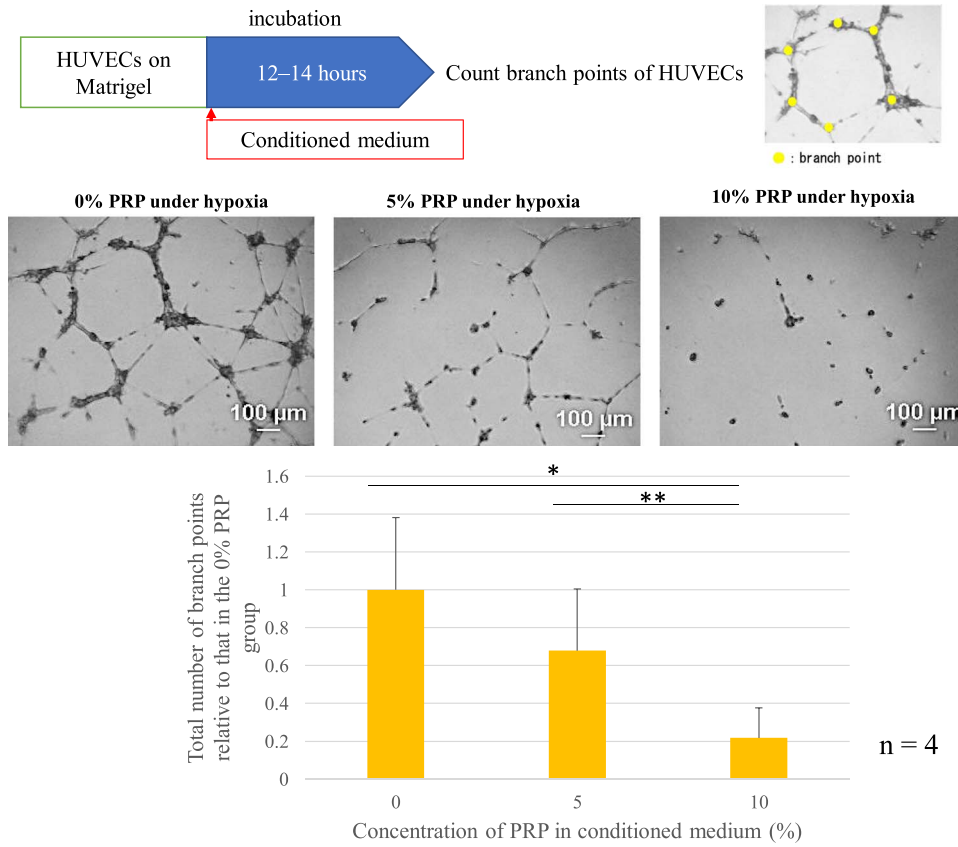

FIGURE 6. Effects of the conditioned medium of PRP-pretreated ADSCs on the tube formation in HUVECs under hypoxia. A, The schematic shows the experimental protocol and the inset shows the branch points of tube formation by HUVECs. Conditioned medium of ADSCs treated with 5% and 10% PRP under hypoxia inhibited the tube formation of HUVECs compared with those treated with 0% PRP. We counted the number of branch points depicted by yellow symbols. B, Graph showing the effects of the conditioned medium of PRP-pretreated ADSCs on the tube formation in HUVECs under hypoxia (n = 4). The number of total branch points was normalized to that in the 0% PRP group. All the measurements were conducted at 24 hours of cultures. *$P < 0.05$, **$P < 0.01$ versus 0% PRP.

the present study, we found that PRP decreased the mRNA expression levels of VEGF, HGF, and TGF-β1, but not bFGF, under hypoxic conditions. Platelet-rich plasma inhibits TGF-β1 expression.[26,27] Because TGF-β1 signaling increases the mRNA levels of VEGF,[28] inhibition of TGF-β1 signaling can cause a decrease in the mRNA levels of VEGF in ADSCs—this idea was confirmed in the present study. A previous report indicated that the downstream signal from TGF-β was different from FGF2-related signal transduction,[29] which suggests that the signal from bFGF could be exceptional. We have not investigated the mechanism underlying the inhibitory action of PRP on the mRNA expression of both TGF-β1 and HGF under normoxia. Platelet-rich plasma with or without leukocytes has different effects on the mRNA levels of TGF-β1 and HGF in synoviocytes. In a previous study, PRP containing leukocytes tended to decrease mRNA of TGF-β1 and significantly reduce mRNA of HGF compared with the negative control (PPP: platelet poor plasma) and reduced mRNA of HGF in a dose-dependent manner, which may be extrapolated to the present finding that PRP reduced the mRNA levels of TGF-β and HGF in ADSC under normoxic conditions.[30]

The decrease in the mRNA level of VEGF in PRP-treated ADSCs might attenuate angiogenesis in vascular endothelial cells. Vascular endothelial growth factor is known to activate the phosphatidylinositol-3 kinase/protein kinase B pathway and increase eNOS expression level in vascular endothelial cells.[31] Nitric oxide increases the permeability of the vessel wall and promotes chemotactic migration of endothelial progenitor cells, mural cells, and hematopoietic stem cells to the site of ischemia, forming a capillary plexus and, ultimately, a mature vessel. Phosphorylated eNOS induced by VEGF signaling binds to calmodulin to accelerate NO production in endothelial cells, contributing to endothelial function.[11] The conditioned medium from PRP-treated ADSCs under hypoxic conditions inhibited eNOS phosphorylation in HUVECs, decreased NO production, and attenuated tube formation by HUVECs. This concurs with a previous report indicating a decrease in blood NO levels upon the local injection of PRP and ADSCs.[32] Local injection of PRP and ADSCs in combination enhances wound healing in patients with diabetes mellitus, suggesting that simultaneous administration of PRP and ADSCs may improve the production of VEGF in diabetic ulcers.[12,17] However, the present study suggests that cultured ADSCs exposed to PRP might secrete low levels of angiogenic factors compared with the amounts produced by either ADSCs transplantation alone or simultaneous administration of PRP and ADSCs. eNOS phosphorylation in endothelial cells is positively regulated by PI3/AKT pathway and negatively regulated by the PKC pathway. Hypoxia is known to promote the PKC-dependent signaling pathway in cardiovascular system.[33] PKC has been reported to phosphorylate Threonin[495] of eNOS and inhibit eNOS in endothelial cells.[11] Taken together with the downregulation of VEGF production in ADSCs by PRP, hypoxic conditions may augment the

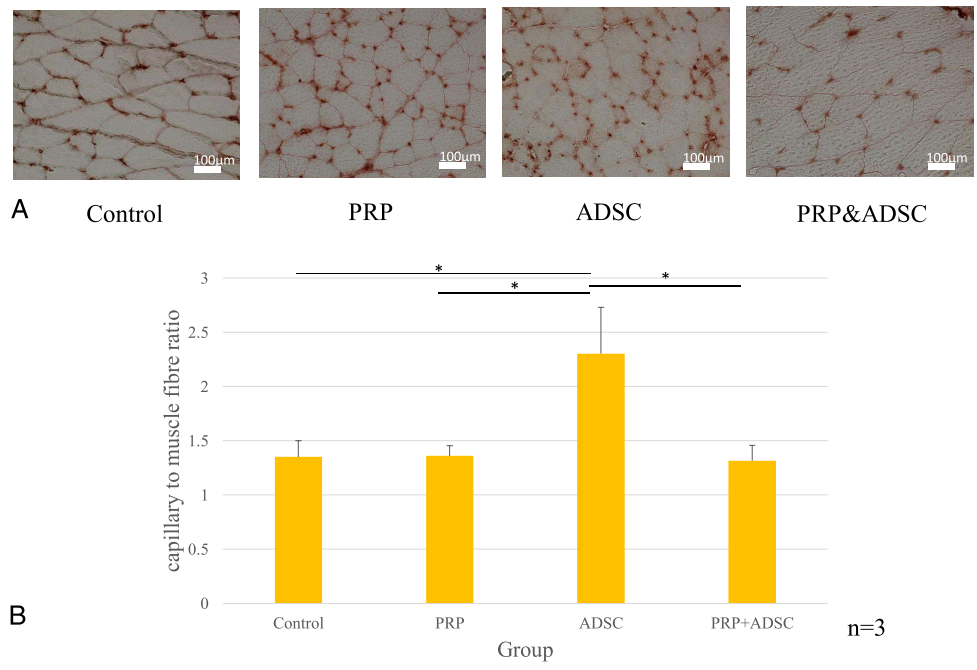

FIGURE 7. Effects of injecting PRP and ADSCs in combination on capillary density in the ischemic limb. A, Representative optical micrographs of capillaries in the musculature of the ischemic limb of Balb c-nu/nu mice, treated with saline (control group), rat PRP (PRP group), rat ADSC alone (ADSC group), rat PRP, and ADSC (PRP + ADSC group). B, Averaged capillary densities as a ratio relative to skeletal muscle density in ischemic hind limbs of Balb c-nu/nu mice. Each bar represents the mean ± S.D. Control group: given saline (n = 3); PRP group: PRP injection group (n = 3); ADSC group: ADSC transplanted group (n = 3); and PRP + ADSC group: ADSC transplanted with PRP injection group (n = 3). *$P < 0.05$ versus ADSC group.

inhibition of eNOS activity in endothelial cells via activation of PKC, resulting in the reduction of NO production by endothelial cells. Further experiments are required to clarify this point.

Although the precise mechanism involved in the attenuation of angiogenesis and NO production induced by conditioned medium from PRP-treated ADSCs remains to be elucidated, its clinical implications are obvious. A decrease in NO production in vascular endothelial cells has been reported to impair their function and blood perfusion in tissues,[34] leading to refractoriness of diabetic skin ulcers.[12] Although PRP and ADSCs are expected to improve angiogenesis and endothelial function, pretreatment of ADSCs with PRP may attenuate ADSC-induced angiogenic actions. Thus, our in vivo data showed that the ADSC transplanted with PRP injection did not improve the blood flow or capillary density in the ischemic limb, whereas the transplanted ADSCs alone significantly improved the blood flow and capillary density. This result supports our hypotheses. In the present study, we found that pretreatment with PRP abolished the angiogenic action of ADSCs. The interpretation of the current results shows that when PRP-supplemented ADSCs is applied to vascular endothelial cells under ischemic conditions, the expected angiogenic effects could get inhibited. Because several clinical trials have demonstrated the angiogenic effects of ADSCs in critical limb diseases, ADSCs could be applied to critical limb diseases.[35] However, this study suggests that combining PRP and ADSCs should be avoided in critical limb diseases as it could inhibit the angiogenic effects of ADSCs. Further studies are necessary on this subject.

There are several limitations to this study, which are as follows: (1) we did not measure the protein levels of VEGF in the conditioned medium of PRP-treated ADSCs but measured their mRNA levels and cellular protein levels of VEGF in ADSCs. In a previous study, PRP significantly reduced VEGF levels.[36] We found that PRP reduced the cellular levels of VEGF in ADSCs (Supplemental Fig. 1, http://links.lww.com/SAP/A785), suggesting that PRP also reduced the expression level of VEGF. We also found that the conditioned medium from PRP-treated ADSCs reduced the levels of phosphorylated VEGF receptors in HUVECs (Supplemental Fig. 2, http://links.lww.com/SAP/A786), which indicates that this conditioned medium might inhibit the signal transduction of VEGF in endothelial cells; (2) we did not examine the details of the concentration-dependent action of ADSCs pretreated with PRP; (3) we did not examine the time-dependent effects of ADSCs pretreated with PRP; and (4) we did not conduct experiments using the same species, because immunostaining using anti-CD 31 antibody does not work in rats.[37]

ACKNOWLEDGMENT

The authors thank Mrs Yumiko Inoue for providing technical support. The authors also thank Editage (http://www.editage.com) for the English language editing.

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