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The effect of DPP-4 inhibitor on angiogenic regeneration by bone marrow mesenchymal stem cell in hind limb ischemia injury model

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Molecular Biology

The effect of DPP-4 inhibitor on angiogenic regeneration by bone marrow mesenchymal stem cell in hindlimb ischemia injury model

Directed by professor Young-Guk Ko

The Master's Thesis Submitted to the

Department of Science for Aging,

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in partial fulfillment of the requirements for the

degree of Master of Science for Aging

Heejung Lim

June 2015

This certifies that the Master's Thesis of

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Hee Jung Lim

June 2015

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ABSTRACT

The effect of DPP-4 inhibitor on angiogenic regeneration by bone marrow mesenchymal stem cell in hindlimb ischemia injury model

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(Directed by Professor Young-Guk Ko)

Background: Mesenchymal stem cells (MSCs) are known to have a therapeutic potential for severe limb ischemia. However, poor survival of implanted MSCs in the target tissue remains as an important factor that attenuates the angiogenic

potential of the cell therapy.

Thus, we investigated whether sitagliptin, a DPP-4 inhibitor, may enhance the angiogenic efficacy of MSC in a hind limb ischemia murine model by increasing production of SDF-1.

Methods and Results: Mice with induced hind limb ischemia were devided into 4 groups; group 1 treated with oral saline and local injection of saline, group 2 treated with oral sitagliptin and local injection of saline, group 3 treated with oral saline and local injection of MSCs, and group 4 treated with oral sitagliptin and local injection of MSCs. Angiogenic responses were measured by laser-Doppler perfusion imaging, muscle capillary density, and protein and mRNA expression of SDF-1, CXCR4, and vascular endothelial growth factor VEGF growth factors and compared among the treatment groups.

The combined treatment of oral sitagliptin and local injection of MSCs achieved more effective angiogenic response than oral sitagliptin administration or local MSC transplantation alone in a mouse hind limb ischemia model. The combination therapy also demonstrated increased expression of VEGF, SDF-1 and CXCR4 in hind limb ischemia models.

Conclusion: The combination therapy of oral sitagliptin and local

transplantation of MSCs was more effective in enhancing angiogenic responses to ischemia than oral sitagliptin or local transplantation of MSCs alone possibly due to up-regulation of SDF-1.

Key Words: Hind-limb ischemia, sitagliptin, SDF-1/CXCR4, angiogenesis, Laser Doppler

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I. INTRODUCTION

Critical limb ischemia (CLI), disease known to affect quality of life and survival, is the most severe form of occlusive atherosclerosis. It is a terminal

peripheral artery disease and presents with chronic ischemic pain, ulcers and/or tissue gangrene [1-4]. These patients frequently have multiple levels of vascular obstruction making collateral blood vessel formation difficult. When endovascular or surgical treatment fails or is not feasible, there are no other known therapies that may effectively rescue the ischemic limb.

Mesenchymal stem cells (MSC) are pluripotent stem cells that can differentiate into osteoblasts, chondrocytes, adipocytes, neurons, skeletal muscle cells, endothelial cells and vascular smooth muscle cells [3,5-10]. They are also known to secrete various cytokines that promote angiogenesis and vasculogenesis. Furthermore, MSCs are considered advantageous for clinical application of cell therapy since they do not induce immunological responses and therefore do not need main histocompatibility match for allogeneic transplantation [9,11]. Therapeutic angiogenesis or vasculogenesis is a treatment targeted to induce new blood vessel formation for improved perfusion of ischemic tissues [12,13] and has a promising potential for the treatment of CLI. Several studies reported that transplanted MSCs induced angiogenesis and improved blood flow to ischemic limbs in rat models of hind limb ischemia [7,14,15]. However, due to the local hypoxia, oxidative stress and inflammation in

the target ischemic tissue, the survival of transplanted MSCs is poor and the therapeutic effects remain attenuated. Thus, it is critical to find techniques to enhance survival of transplanted MSCs in the target tissue.

Stromal-derived factor (SDF)-1, is also known as CXCL12, a potent chemoattractant of stem cells and progenitor cells. SDF-1 is mainly expressed by bone marrow stromal cells and endothelial cells and its production is increased under ischemic conditions. SDF-1 plays a significant role in the process involving stem/progenitor cell chemotaxis and organ-specific homing in ischemic tissue through interaction with its receptor CXC chemokine receptor 4 (CXCR4) on the surface of stem/progenitor cells. Administration of SDF-1 has shown to increase the number of circulating endothelial progenitor cells (EPCs) and to improve endothelial function [16,17]. SDF-1 also improved cardiomyocyte survival, neovascularization, and cardiac function after myocardial infarction [18-21].

Recent studies reported that oral sitagliptin, an inhibitor of dipeptidyl peptidase-4 (DPP-4) was able to increase circulating endothelial progenitor cells possibly by up-regulation of SDF-1. DPP-4 is known to cleave various bioactive molecules such as SDF-1 and glucagon-like peptide-1 (GLP-1) [16,22,23].

Therefore, the purpose of this study is to investigate whether combined treatment of oral sitagliptin and local injection of MSCs may have synergistic effects on angiogenesis and blood perfusion recovery in a murine hind limb ischemia model by up-regulation of SDF-1.

II. MATERIALS AND METHODS

2.1. Reagents

Sitagliptin (Januvia®) was purchased from Merck. Anti-VEGF (1:1000), Anti-SDF-1 (1:1000), and Anti-CXCR4 (1:1000) antibodies were obtained from Abcam (Cambridge, MA, USA), and Anti-GAPDH was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

2.2. Animals

All female C57BL/6 mice at 7-week-old (20-22g body weight) were purchased from Orient Bio (Sungnam, Korea). A total of 28 mice were devided into 4 groups; group 1 treated with oral saline and local injection of saline, group 2 treated with oral sitagliptin and local injection of saline, group 3 treated with oral saline and local injection of MSCs, and group 4 treated with oral sitagliptin and local injection of MSCs. Experiments were approved by the Institutional Animals Care and Use Committee of Yonsei University College of Medicine and performed in accordance with "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3. Cell Culture

Human mesenchymal stem cells (hMSCs) were obtained from Lonza Walkersville Inc. (Walkersville, MD, USA). The hMSCs were maintained under DMEM-low glucose (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin at 37℃ humidified atmosphere containing 5% CO_2-95% air. DMEM was refreshed every one day until sub-confluent. All experiments were performed using cells between passage numbers 5 to 7.

2.4. Mouse Hind limb Ischemia

The mice were anesthetized by an intraperitoneal injection of 0.05mg/kg (Zoletile Virbac, Carros Cedex, France) and xylazine 0.15ml/kg (Rompen, Bayer, Leverkusen, Germany). After the skin incision, the proximal and distal portions of the external femoral artery and all of the above saphenous artery was ligated. The femoral arterial and all side branches artery were dissected and excised [20].

2.5. Sitagliptin Administration and hMSC Transplantation

Sitagliptin (Januvia®) was obtained from Merck Sharp & Dohme Corp Korea. Sitagliptin 20 mg/kg body weight or 500 μl of saline per mouse was

administered daily after the onset of ischemia using oral gavage. Local injection of MSC (10^6 cells/200 μl) or saline 200 μl into the ischemic thigh muscle of four different sites was performed using with a 26 gauge needle under local anesthesia at 3 days after induction of the hind limb ischemia. Sitagliptin (20mg/kg BW), or saline was orally administered for 28day.

2.6. Measurement of Laser Doppler Blood Perfusion Imaging

A laser-Doppler image system (Moor LDI2, Moor Instruments, Axminster, UK) was used to measure blood flow perfusion before and at 7, 21, and 28 days after limb ischemia surgery. Before imaging, excess fur were removed from the limbs using depilatory cream, and mice were placed on a temperature controller at 37℃. Relative blood perfusion data were expressed as the ratio of the ischemic (left) to non-ischemic (right) limb blood flow.

2.7. Histological Assessment for Capillary Density

Ischemic limb gastrocnemius muscle were harvested 28 days after induction of ischemia, immersion-fixed with 4% buffered paraformaldehyde, and subsequently embedded in paraffin. Rabbit anti-mouse CD31 (dilution 1:200; polyclonal; Abcam) was used to determine the capillary density on 2-μm-thick paraffin-embedded sections using standard immunofluorescence. This measurement was determined in three randomly selected low-power (original

magnification x200) fields from each animal, and the average value was used as a single data for each animal.

2.8. Immunoblot analysis

For immunoblotting, homogenates and sonicator of limb muscle tissue were analyzed. Equal amounts of proteins were loaded and separated in 10%, 12.5% and 15% SDS-polyacrylamide/bis-acrylamide gel electrophoresis and transferred to poly vinylidene difluoride membrane (Bio-Rad Laboratories, Inc. Hercules, CA, USA). The membrane was blocked for 1hr by 10% skim milk. Thereafter, the membrane was washed 3 times with TBS-tween 20 (TBS-T, 0.1% tween 20) for 7 min at room temperature. Membrane was incubated with primary antibodies for overnight at 4℃. Membrane was washed five times with TBS-T for 7 min, and incubated for 1hr at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the bands were evaluated.

2.9. Reverse transcription polymerase reaction (RT-PCR)

Total RNA from frozen muscle was isolated using the Qiazol-Reagent. cDNA synthesis was then performed using 1ug of RNA with the TaKaRa Ex Taq™ polymerase (Takara Bio, Otsu, Shiga, Japan). Levels of mRNA were analyzed by RT-PCR using the primers shown in Table 1. PCR products were

separated by electrophoresis in a 1.2% agarose-gel containing Gel-red (Biotium, Hayward, CA, USA).

Table 1. PCR primers used in this study

Gene	Primer	Size(bp)	Temp.(℃)
VEGF	5'- GTA CCT CCA CCA TGC CAA GT -3'	340	58
	5'- GCA TTC ACA TCT GCT GTG CT -3'		
SDF-1	5' -GCT CTG CAT CAG TGA CGG TA -3'	306	58
	5'- CTT TTC TGG GCA GCC TTT CT -3'		
CXCR4	5'- TCC TGC CCA CCA TCT ACT TC -3'	342	58
	5'- TTT CAG CCA GCA GTT TCC TT -3'		
GAPDH	5'- ACT CCA CTC ACG GCA AAT TC-3'	370	58
	5'- CCT TCC ACA ATG CCA AAG TT -3'		

2.10. Statistical Analysis

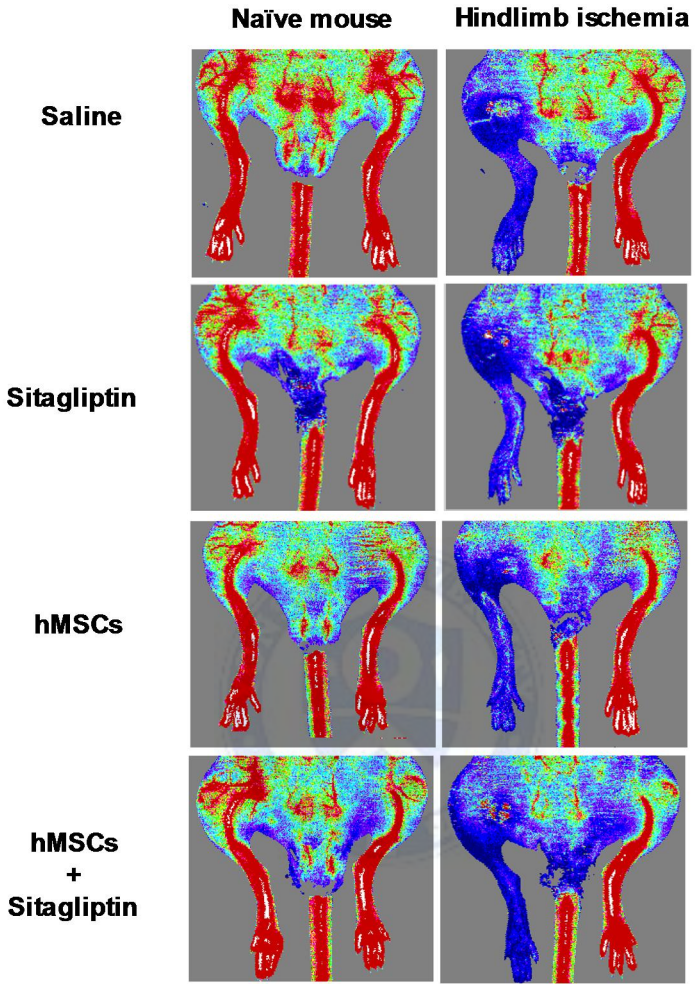
Statistical analysis was performed using IBM PASW Statistics 20.0 software (IBM Corp, Armonk, NY, USA). All results are expressed as means ± standard deviation. Comparisons of continuous variables among the groups were performed using Student's t test or ANOVA. A P value of <0.05 was considered statistically significant.

III. RESULTS

1. Sitagliptin-hMSCs administration promoted reperfusion of blood flow in the ischemia limbs

Representative images of laser Doppler perfusion scan before and immediately after induction of hind limb ischemia were shown in Figure 1A. Serial follow-up images of laser Doppler perfusion scan at 7, 14, 21, and 28 days were presented in Figure 1B. At 28 day, group 4 with combined therapy of oral sitagliptin and local hMSCs showed the highest blood perfusion ratio (0.60 ± 0.1; $P < 0.05$ vs. other groups). The blood perfusion ratio in group 2 and 3 was 0.48 ± 0.08 and 0.55 ± 0.09, respectively. These values were significantly higher than that of group 1 (0.44 ± 0.12, $P < 0.05$). However, the combined use of sitagaliptin-hMSCs further improved angiogenesis (perfusion ratio of). There was no significant difference in blood flow for sitagliptin administration and saline between days 7 and 28 (Figure 1C).

A



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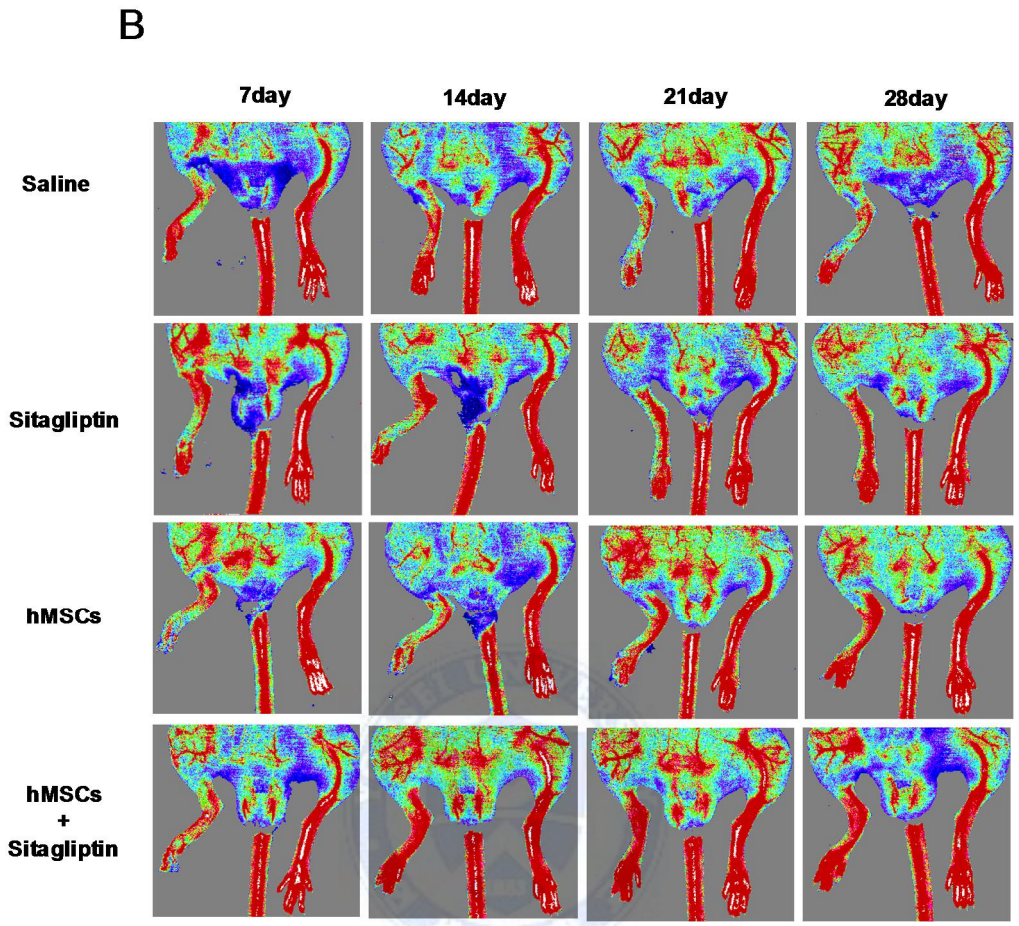

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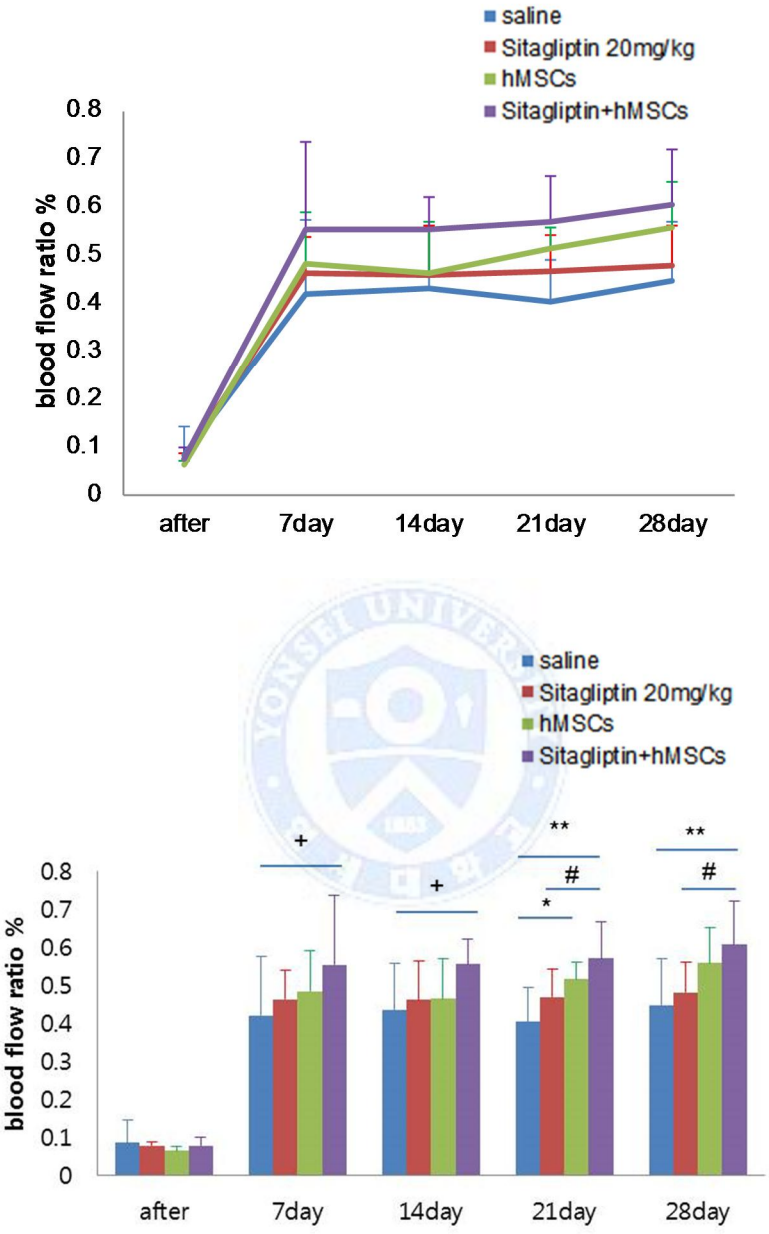

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Figure 1. Femoral artery blood flow by laser-Doppler perfusion imaging in combination of sitagliptin-hMSCs increases neovascularization in hind limb ischemia. (A) Representative results of laser Doppler perfusion imaging before and immediately after induction of hind limb ischemia (left, ischemic; right non-ischemic hind limbs); (B) Representative results of laser Doppler perfusion imaging at 7, 14, 21 and 28days after hind limb ischemia surgery. In color-coded images, normal blood perfusion is displayed as *red*, whereas low or absent blood perfusion is displayed as *blue*; (C) Laser-Doppler ischemic/non-ischemic limb blood perfusion ratios. (**P<0.01 for saline vs. sitagliptin + hMSCs; +p<0.05 for saline vs. sitagliptin + hMSCs; *p<0.01 for saline vs. hMSCs, #p<0.05 for sitagliptin vs. sitagliptin + hMSCs)

2. Sitagliptin-hMSCS improve angiogenesis in ischemic hind limb

Immunohistochemical staining of the shows CD31- and DAPI-positive cells (Figure 3A). The capillary density was significantly higher in group 3 (hMSCs) and 4 (sitagliptin-hMSCs) than in group 1 (saline) or 2 (sitagliptin) (*$p < 0.01$ vs. saline group; ▲$p < 0.01$ vs. Sitagliptin; #$p < 0.01$ vs. MSC) (Figure 3B). There was no significant difference in capillary density between group 3 and 4. However, there was a trend toward a higher capillary density in group 4.

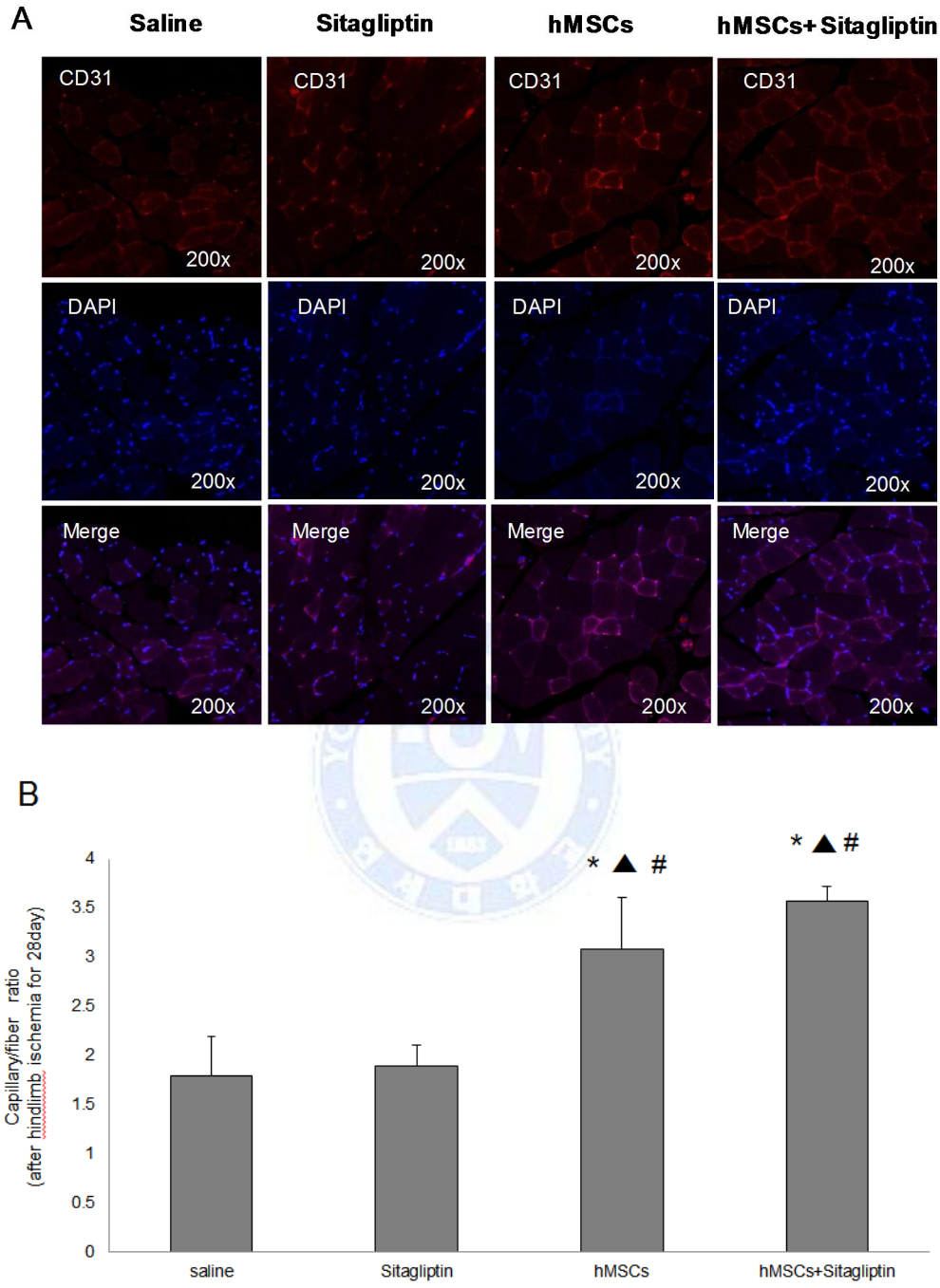

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Figure 2. Effects of sitaglitin-hMSCs on angiogenesis in ischemic limbs. (A) Capillaries (*re*d) were identified by CD31staining, a marker for endothelial cells. CD31 was also observed with confocal microscopy using primary anti-PECAM1 antibody and Alexa-conjugated secondary antibody. (B) Quantitatively expressed as a capillary number per muscle fiber on 28days after operation (n=3; for each experimental group, original magnification, x200).

3. Sitaglipin-hMSCs increases VEGF expression in hind limb ischemia muscle

Representative Western blot data of VEGF protein expression are shown in Figure 3. There was increased VEGF expression in group 2 and group 4 compared to group 1 or group 3 (Figure 3A). Similarly to protein expression, that total RNA expression of VEGF was higher in group 2 and 4 group compared to that in group 1 and 3 (Figure 3B)

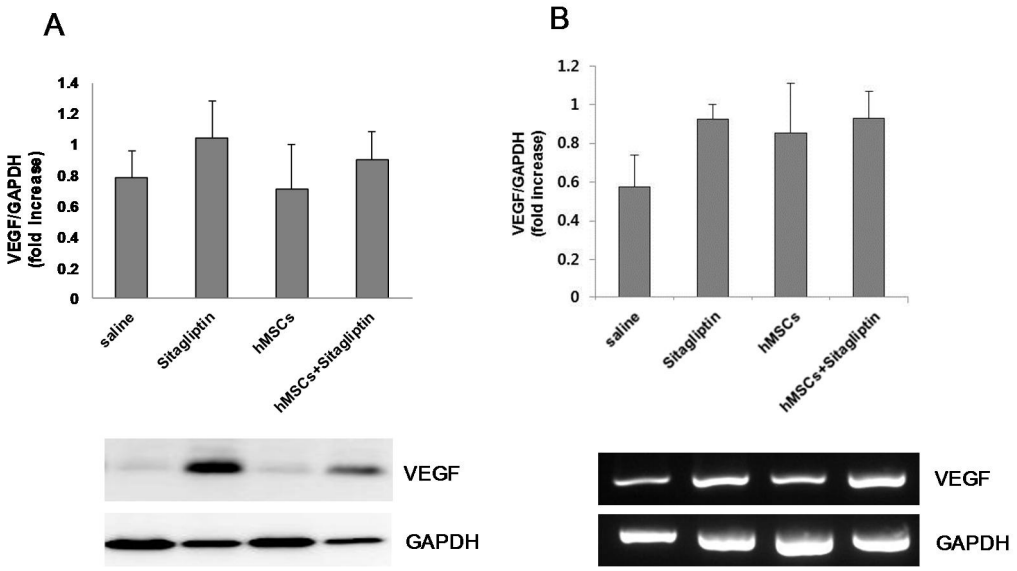

Figure 3. Effects of sitagliptin-hMSCs on VEGF expression levels hind limb ischemia injury model. (A) Gastrocnemius muscle tissues were collected at 7 days after hind-limb ischemia injury. Muscle tissue lysates were separated by SDS-PAGE gel and analyzed by immunoblot analysis with antibodies against anti-VEGF and anti-GADPH antibody. (GADPH was loading control n=4). The protein expression of VEGF was slightly increased in group 2 (sitagliptin) and group 4 (sitagliptin-hMSCs) than in group 1 (saline) and group 3 (hMSCs). (B) Total RNA was analyzed by Reverse transcription-polymerase chain reaction

(RT-PCR) using primers specific of VEGF and GAPDH gene in ischemic muscle tissues (GAPDH was used as the internal control. n=4). The total RNA expressions of VEGF was slightly increased in group 2 (sitagliptin) and group 4 (sitagliptin-hMSCs) than in other groups.

4. Sitaglin-hMSCs increases SDF-1 expression and secretion in hind limb ischemia

Western blot data show no significant difference in SDF-1 protein expression among the groups (Figure 4A). However, there was increased RNA expression of SDF-1 in group 3 and 4 compared to that of group 1 and 2. However, SDF-1 RNA expression was similar between group 3 and 4 (Figure 4B). Interestingly, western blot analysis using serum showed significantly increased protein level of SDF-1 in group 4 compared with other groups (Figure 4C).

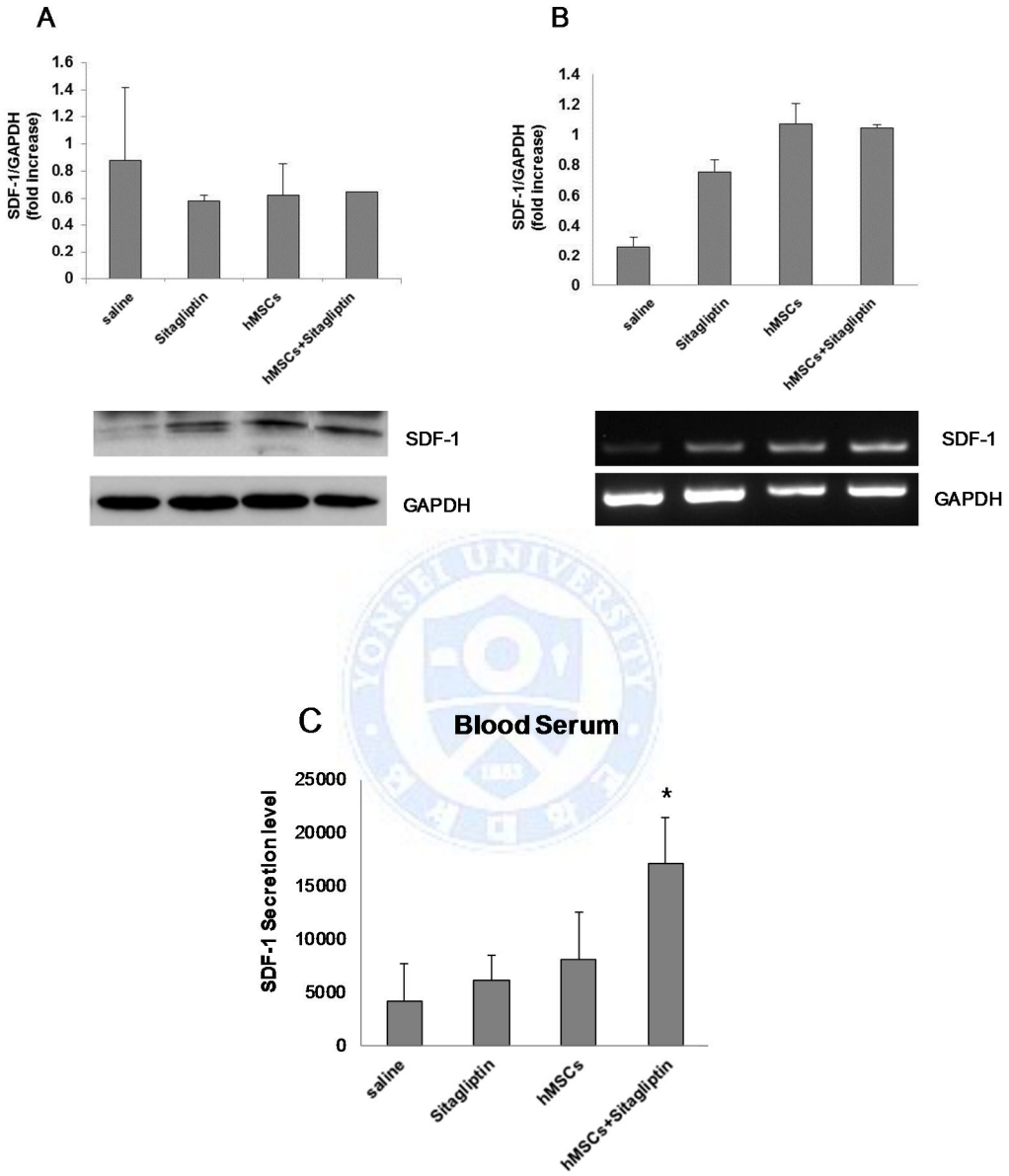

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Figure 4. Effects of sitagliptin-hMSCs on SDF-1 expression levels in hind limb ischemia model. (A) Representative Western blot of SDF-1 protein level in the gastrocnemius muscle tissues at 7 days after limb ischemic injury (n=2). The protein expression of SDF-1 was lower in group 1 (saline) than in other groups. (GADPH was used as the internal control. n=2). (B) RNA was analyzed by Reverse transcription-polymerase chain reaction (RT-PCR) using primers specific of SDF and GAPDH gene in ischemic muscle tissues. (GAPDH was used as the internal control. n=4). (C) The protein expression of SDF-1 was significant higher in sitagliptin-hMSCs combination therapy group than in other groups (n = 4) $*P < 0.01$

5. Sitagliptin increases CXCR4 expression in hind limb ischemia muscle

There was increased expression level of CXCR4 in group 2 and 4 than in group 1 and 3 (Figure 5A). RNA expression level of CXCR4 similarly increased in group 2, 3, and 4 was higher than in group 1 (Figure 5B).

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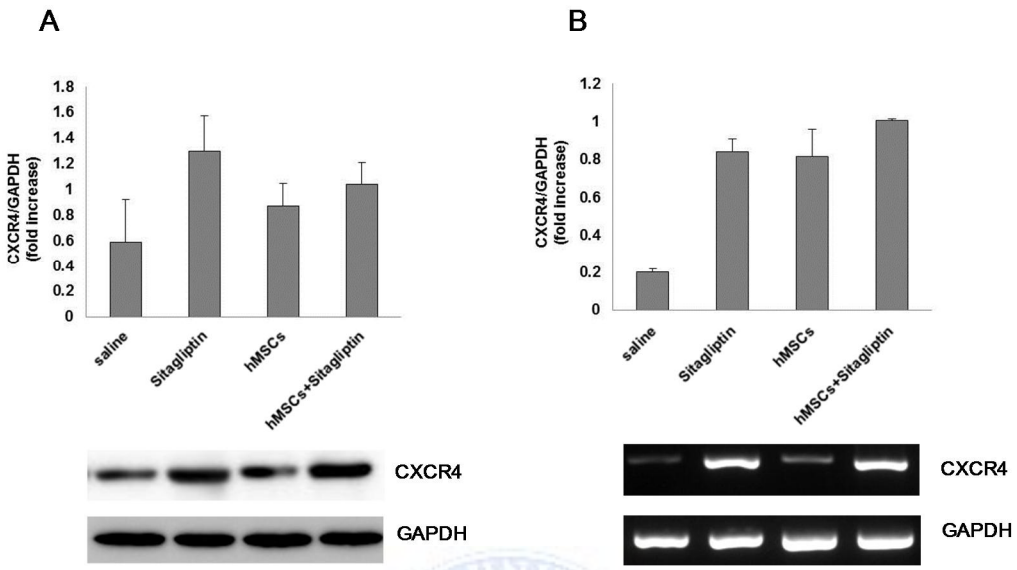

Figure 5. Effects of sitagliptin on CXCR4 expression levels in hind limb ischemia. (A) Gastrocnemius muscle tissues were collected 7 days after hind-limb ischemia injury. Muscle tissue lysates were separated by SDS-PAGE gel and analyzed by immunoblot analysis with antibodies against anti-CXCR4 and anti-GADPH antibody. (GADPH was used as the internal control. n=4). The protein expression of CXCR4 was increased in group 2 (sitagliptin) and group 4 (sitagliptin-hMSCs) than in group 1 (saline) and group 3 (hMSCs). (B) RNA was analyzed by Reverse transcription-polymerase chain reaction (RT-PCR) using primers specific of CXCR4 and GAPDH gene in ischemic muscle tissues

(GAPDH was used as the internal control. n=4). The RNA expression of CXCR4 was significantly higher in group 2 (sitagliptin) and group 4 (sitagliptin-hMSCs).

IV. DISCUSSION

The major finding of study was that combined treatment of oral sitagliptin and local injection of MSCs achieved more effective angiogenic response in a mouse hind limb ischemia model than oral sitagliptin administration or local MSC transplantation alone. The combination therapy was associated with increased expression of VEGF, SDF-1 and CXCR4.

Mesenchymal stem cell (MSC) is a multipotent stem cell that can differentiate into a variety of cell types. Various studies demonstrated therapeutic angiogenic effects of MSCs in animal models of peripheral arterial disease and myocardial infarction [5,15,24,25]. However, the mechanisms underlying the effects of MDSCs remain still unclear. Stem cell can either directly increase angiogenesis by transforming into endothelial cells and proliferation or indirectly by secreting various angiogenic growth factors and anti-apoptotic factors. But, there are several shortcomings in using MSCs for cell therapy of ischemic diseases. The repair capability of MSCs declines with age and diseases [24,26-29]. Also, the viability of MSCs in ischemic condition is low. Thus, it is crucial to improve MSC survival against the hypoxia and hypertrophic microenvironment [30-33]. There have been various studies focused

on treatment strategies to improve survival of transplanted MSCs in the target organs [34-37].

Sitagliptin, an anti-diabetic drug, is an inhibitor of DPP-4. DPP-4 cleaves various substrates, including GLP-1 and SDF-1. Increased activity of DPP-4 in blood has shown to decrease the circulating concentration of SDF-1a [38]. SDF-1 has been found to be involved in mobilization and recruitment of endothelial progenitor cells after arterial injury in mice [39,40]. Up-regulation of SDF-1, CXCR4, and VEGF in the damaged tissue has been shown to play a critical role in recruiting stem cells to ischemic tissue [19,41,42]. Another experiment study demonstrated that SDF-1 pretreatment can improve MSC migration, cytokine production, and cell survival after exposure to hypoxia [43-45]. In our study, expressions of SDF-1, CXCR4, VEGF were increased in all animals treated with oral sitagliptin, local transplantation of MSCs, or the combined treatment compared with control group. However, we could not prove whether the expression of these angiogenic factors was statistically higher in the combination group than in the monotherapy of sitagliptin or MSCs due to small numbers of study animals. However, there was signicantly improved angiogenic response measured in blood flow ratio by laser Doppler perfusion scan and capillary density for the combination therapy group than monotherapy of oral sitagliptin or local transplantation of MSCs. Therefore, we

assume that additive effects of sitagliptin and MSCs may be achieved by up-regulation of SDF-1/CXCR4 axis and activation of MSCs.

A major limitation of the present study was that insufficient data to prove working mechanisms of the combined therapy of sitagliptin and MSCs for improved angiogenic response Furthermore, we could not also demonstrate prolonged survival or increased activity of MSCs in ischemic limbs.

V. CONCLUSION

The combination therapy of oral sitagliptin and local transplantation of MSCs was more effective in enhancing angiogenic responses to limb ischemia than oral sitagliptin or local transplantation of MSCs alone possibly due to up-regulation of SDF-1. Our results suggest that this combined treatment may have a potential as a new therapeutic strategy to treat severe peripheral arterial disease.

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ABSTRACT (in Korean)

하지허혈 모델에서 골수유래 중간엽줄기세포의 혈관재생효과에 미치는 DPP4 저해제의 영향에 관한 연구

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임 희 정

Background: 중간 엽 줄기 세포는 중증 하지 허혈에 대한 치료 가능성을 갖는 것으로 알려져 있다. 그러나 허혈 조직에 중간 엽 줄기 세포 주입이 낮은 생존율로 인해 줄기세포 치료의 혈관 신생을

약화 시키는 것이 중요한 요인이다. 따라서 우리는 sitagliptin, DPP-4 억제제가 SDF-1 의 발현에 의해 하지 허혈 쥐 모델에서 중간 엽 줄기세포의 혈관 신생 반응을 향상 시킬 수 있는지 알아보고자 한다.

Methods and Results: 하지 허혈 유도 후 네 군으로 분류하였다. 각각 Group 1 saline 경구 투여, group 2 는 sitagliptin 경구 투여, group 3 saline 경구 투여 및 중간 엽 줄기 세포의 주입, group4 sitagliptin 경구 투여 및 중간 엽 줄기세포 주입으로 나누어 진행하였다. 레이져 도플러 관류 영상을 통해 혈관 신생을 측정하였으며, 근육 모세관 밀도와 SDF-1, CXCR4, VEGF 같은 혈관 신생 인자의 mRNA 와 단백질 발현을 알아보았다. 하지 허혈 모델에서 Sitagliptin 과 중간 엽 줄기 세포 동시 투여 군이 sitagliptin 단독 투여 군 또는 hMSCs 단독 주입 군에 비해 혈관 신생 효과가 있는 것을 확인하였다. 또한 Sitagliptin 과 중간 엽 줄기세포 동시 투여 치료법은 하지 허혈 모델에서 VEGF, SDF-1, CXCR4 의 발현이 증가하는 것을 입증하였다.

Conclusions: 하지 허혈에서 Sitagliptin 과 중간 엽 줄기 세포의 동시 투여는 sitagliptin 단독 투여와 중간 엽 줄기 세포 단독 주입

치료법보다 혈관 신생 반응을 촉진한다. 이는 SDF-1 과발현에 의해 혈관 신생을 촉진 되는 것을 확인하였다.

핵심되는 말: 하지 허혈, sitagliptin, SDF-1/CXCR4, 혈관신생반응