Combination of exendin-4 and DPP-4 silencing promoted angiogenesis of human coronary artery endothelial cells via activation of PI3K/Akt pathway

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Abstract: This study was aimed to explore the combined effects of Exendin-4 with dipeptidyl peptidase-IV (DPP-4) silencing on vascular endothelial growth factor (VEGF)-induced cell proliferation and angiogenesis in Human Coronary Artery Endothelial Cells (HCAECs), as well as the underlying molecular mechanisms which were involved in this process. HCAECs were treated by exendin-4, small interfering RNA (siRNA) targeting DPP-4 (DPP-4-siRNA) or exendin-4 plus DPP-4-siRNA, respectively. Cell migration, proliferation and angiogenesis *in vitro* were assessed by scratch-wound assay, MTT, tran swell assay, and matrigel tube formation, respectively. Cell apoptosis was investigated by TUNEL assay. Expression of apoptosis and PI3K/Akt related proteins were assessed by Western blotting. Incubation of HCAECs with exendin-4 and silencing of DPP-4 both caused an increase in cell proliferation, migration and tube formation, while a significant decrease in apoptosis (all $p<0.05$). Furthermore, the combination of the exendin-4 and silencing of DPP-4 had additional effects on HCAECs. Protein levels of p-Akt and p-PI3K were markedly increased by exendin-4 incubation, silencing of DPP-4 in HCAECs. These results suggest that combination of exendin-4 and silencing of DPP-4 had additional promoted effects on angiogenesis of HCAECs via activation of PI3K/Akt pathway. Our study indicated an alternative therapeutic strategy for atherosclerotic neovascularization.

Keywords: Exendin-4, Dipeptidyl peptidase-IV, angiogenesis, HCAEC, PI3K/Akt.

INTRODUCTION

Atherosclerosis diseases, which contribute to one-third of deaths worldwide yearly, have been consistently regarded as a major cause of disability and mortality (Buldak *et al*., 2016; Calles-Escandon and Cipolla, 2001). Pathological neovascularization is a prominent feature of atherosclerotic lesions (Carmeliet, 2003; Cheng *et al*., 2013), while inappropriate angiogenesis improves prognosis of neovascularization involved in atherosclerosis (Chistiakov *et al*., 2015). The pathological angiogenesis of atherosclerosis are responsible for promotion of plaque expansion, plaque vulnerability, endothelialization, intraplaque hemorrhage (IPH), and plaque rupture (de Vries and Quax, 2016; Di Stefano *et al*., 2009; Drucker and Nauck, 2006). Therefore, it is important to understand mechanisms underlying aberrant angiogenesis of atherosclerosis, and this will enable development of innovative and alternative atherosclerosis therapies.

Endothelial cells play a pivotal role in angiogenesis, which regulates both neovascularization and the integrity of intimal layer (Erdogdu *et al*., 2012). Both apoptosis and growth of endothelial cells influenced endothelial repairing and its normal function. The vascular endothelial disruption induces abnormal vasomotion, promotes aberrant adhesion of cells, including platelets and monocytes, which finally causes proliferation and migration of vascular smooth muscle cells (Hotamisligil, 2010). Moreover, such pathological changes promoted coagulation, aberrant cell adhesion, activated trans-endothelial transport of atherogenic lipoproteins, inflammation and inappropriate vasodilation or vasoconstriction (Jeziorska and Woolley, 1999).

As a brain-gut insulinotropic peptide, glucagon-like peptide-1 (GLP-1) plays an important role in the regulation of glucose homeostasis. Moreover GLP-1 has been used as a clinical treatment for diabetes (Kawasaki *et al*., 2003; Li *et al*., 2016). Native GLP-1 can be rapidly degraded by dipeptidyl peptidase-IV (DPP-4) with a short half-life (a few minutes). As a stable GLP-1 analogue, Exendin-4 can inhibit DPP-4 (Li *et al*., 2016) and has been approved in both U.S. and Europe for clinical treatment of type 2 diabetes. DPP-4 inhibitor block the degradation of gastric inhibitory peptide (GIP), GLP-1 and a variety of other peptides including brain natriuretic peptide. Current literatures have suggested that DDP-4 inhibitor had promising effect in the local wound healing of diabetic foot ulcers in addition to its previously reported glycaemic control (Moreno *et al*., 2012). However, some studies have indicated that these two treatments might worsen prognosis of atherosclerosis due to their promoting effect on angiogenesis.

Therefore, our present study investigated the combined effects of Exendin-4 and DPP-4 silencing on VEGF-

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induced cell proliferation and angiogenesis in HCAECs, further, we explored underlying molecular mechanism which was involved in this process. Our study may provide new insights into the effective treatment of cardiovascular diseases.

MATERIALS AND METHODS

Cell culture

Human Coronary Artery Endothelial Cells (HCAECs) were purchased from Sciencell (USA) and cultured at 37°C in 5% CO_2 in endothelial basal medium-2 (EBM-2, Clonetics) combined with 5% FBS, 1% penicillin/ streptomycin, and endothelial growth factors. HCAECs were cultured till confluence of cells, then plated with routine protocols for assays.

Exendin-4 incubation and DDP-4 siRNA transfection

To assess multiple effects of Exendin-4 on cell viability, migration, tube formation and apoptosis, HCAECs were cultured to confluence with percentage of 90%, and then incubation was performed overnight in FBS-deficient EGM medium. Then, Exendin-4 was added at concentration of 10nM and maintain incubation for 48 h.

In transfection experiment, HCAECs that were cultured in serum free EBM-2 medium were passaged in 6-well plates. The HCAECs were transfected with 100nM of DDP-4 siRNA (Invitrogen, Carlsbad, CA) binding integrin DPP-4 (5′-ATCGGGAAGTGGCGTGTTCAA-3′) with the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) with routine instructions. Negative control siRNA (Invitrogen, Carlsbad, CA) was synthesized as control. Exendin-4 was added after 6 h of transfection. Treated cells were harvested for further assessment after 48 h transfection. The efficacy of DPP-4 silencing was examined with real-time polymerase chain reaction (RT-PCR) and Western blotting.

Cell proliferation assay

Cell proliferation was assessed with the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay (Erdogdu *et al*., 2012). In short, HCAECs were cultured in a 96-well plate with a density of 3×10^3 cells/well and treated with exendin-4, DDP-4 siRNA and exendin-4 plus DDP-4 siRNA, respectively. Further, the medium was replaced with fresh medium containing 0.5 mg/ml MTT (4 hours). At the end of culture, formazan crystals, which results from MTT reduction, were dissolved by treatment of 150µl dimethyl sulfoxide per well. The absorbance value was assessed at 570nm with an automated ELISA plate reader.

Migration and invasion assay

Quantitative cell migration assays were performed with a modified Boyden chamber (Costar-Corning, New York, USA) with 8.0-µm pore polycarbonate filter inserts in 24-well plates as described previously (Hotamisligil, 2010). In brief, the lower chamber was treated with 500µl of complete medium. And HCAECs (5×10^4 cells per well) resuspended in 300µl of serum-free medium were transferred into the upper chamber for treatment of exendin-4, DDP-4 siRNA and exendin-4 plus DDP-4 siRNA, respectively. The cells were cultured to migrate for 24 h at 37°C. The non-migrated cells were discarded from the upper surface of the membrane through scraping of cotton swabs, then, the migrating cells were fixed with methanol and stained with crystal violet (Beyotime, Nantong, China). All cells were photographed under an inverted fluorescence microscope (Olympus IX51) equipped with an Olympus Qcolor 3 digital camera. Migration was assessed through counting the number of stained cells (10 random fields, × 200 magnification). Cell invasion assay was performed with similar protocol, except that transwell inserts were replaced with matrigel-coated.

Scratch assay

Cells were planted in a 6-well culture plates and incubated overnight to a density of 60%–70% (Hotamisligil, 2010). Cell monolayers pretreated with exendin-4, DDP-4 siRNA and exendin-4 plus DDP-4 siRNA were then scratched with a 100-µL yellow pipette tip and washed with PBS solution three times to remove detached cells. The wounded areas were imaged using an Olympus microscope.

Angiogenesis-tube formation on matrigel

Tube formation was examined with *in vitro* matrigel assays as described previously (Pescetelli *et al*., 2015). 50 µl of matrigel (BD Biosciences) per well was added into a 96-well plate and cultured to solidify for 30 min at 37°C. HCAECs were starved in basal medium (without endothelial growth supplements) for 8 h. Then, the cells were inoculated on matrigel surface and treated with exendin-4, DDP-4 siRNA or exendin-4 plus DDP-4 siRNA, and then intervened with VEGF (20ng/ml) for 18 h. The morphological changes of the cells and tube formation were assessed under a microscope and photographed (200× magnification). Tube formation was quantified by counting the number of connected cells (randomly selected fields, 200× magnification), and calculated the ratio with routine method.

TUNEL staining

The cells were incubated for 24 h and were pre-treated with exendin-4, DDP-4 siRNA or exendin-4 plus DDP-4 siRNA according to protocols mentioned above. Cells were stained for assessment of apoptosis with the terminal deoxynucleotidyl (TUNEL) method. We used a commercially available kit (ApopTag Peroxidase In Situ Apoptosis Detection Kit S7100; Chemicon International, Billerica, Massachusetts, USA) for TUNEL apoptosis. The HCAECs were then rinsed 3 times in PBS solution and assessed under a fluorescence microscope with an appropriate filter.

Western blot analysis

HCAECs were cultured in a 100 mm culture plate and allowed to grow into 80% confluence. After treatment with exendin-4, DDP-4 siRNA or exendin-4 plus DDP-4 siRNA, the total cellular protein was extracted with routine protocol, separated by SDS–PAGE and transblotted with routine PVDF membranes. The membranes were blocked with 5% defatted milk for 2 h and then incubated with specific primary antibodies(PI3K, p-PI3K, Akt, p-Akt and β-actin) overnight at 4°C, followed by treatment with appropriate HRP-conjugated secondary antibody for 2h at room temperature.

STATISTICAL ANALYSIS

GraphPad Prism (GraphPad Software Inc., San Diego, California, USA) was used for statistical analyses. All data are presented as the mean ± SD and were assessed with T test or ANOVA. Significance with $p < 0.05$ was considered statistically different.

RESULTS

Exendin-4 and DDP-4 silence promotes proliferation of human coronary artery endothelial cells

MTT assays showed that, compared with control, Exendin-4 treatment and DDP-4 silence significantly promotes proliferation of HCAEC, while combination of Exendin-4 and DDP-4 silence further promoted proliferation (fig. 1).

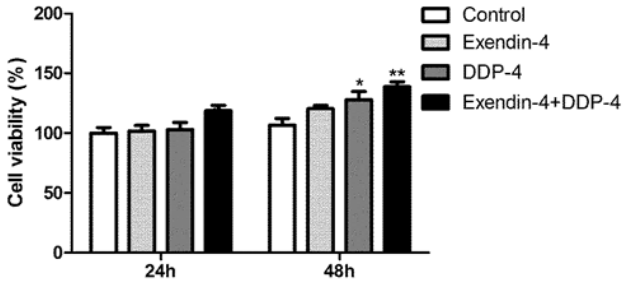

Fig. 1: Effects of Exendin-4 and DDP-4 on HCAECs proliferation. After pretreatment with exendin-4, DDP-4 siRNA or exendin-4 plus DDP-4 siRNA, the effects on HCAECs proliferation was evaluated by MTT. Combined use of exendin-4 and DDP-4 siRNA had the strongest promoted effects on HCAECs proliferation. * $p<0.05$, ** $p<0.01$ compared to the control group.

Exendin-4 and DDP-4 silence promotes cell migration and invasion in human coronary artery endothelial cells

Both cell migration and invasion was enhanced after Exendin-4 treatment of DDP-4 silence (fig. 2A& B). Furthermore, compared with other groups, combination of Exendin-4 and DDP-4 silence had the largest numbers of cell migration and invasion.

Exendin-4 and DDP-4 silence promotes wound healing in human coronary artery endothelial cells

Wound healing was improved by Exendin-4 treatment and DDP-4 silence, verified by scratch assay (fig. 3). Combination of Exendin-4 and DDP-4 silence further promoted wound healing (fig. 3).

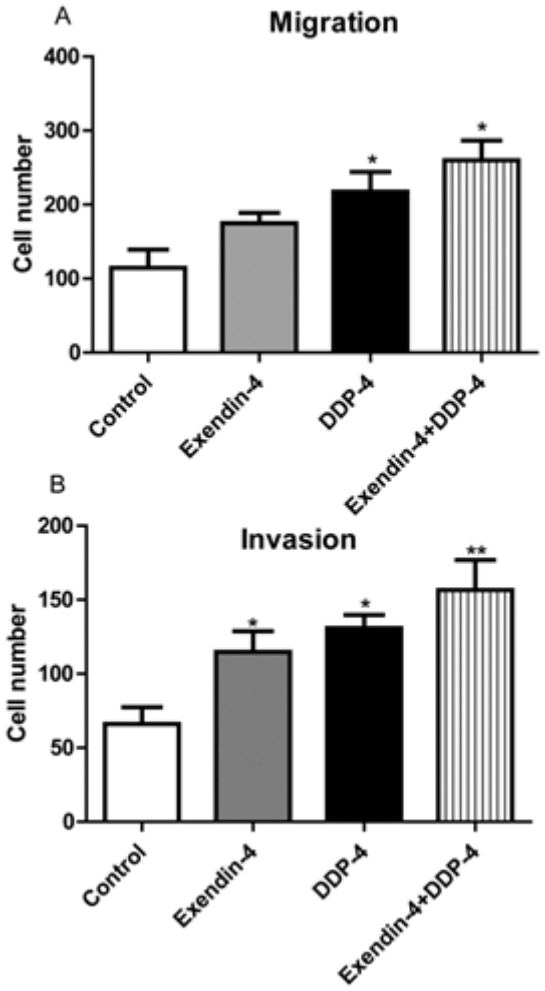

Fig. 2: Effects of Exendin-4 and DDP-4 on HCAECs migration and invasion. After pretreatment with exendin-4, DDP-4 siRNA or exendin-4 plus DDP-4 siRNA, the effects on HCAECs migration (A) and invasion (B) was evaluated by transwell. Combined use of exendin-4 and DDP-4 siRNA had the strongest promoted effects on HCAECs migration and invasion.*$p<0.05$, **$p<0.01$ compared to the control group.

Exendin-4 and DDP-4 silence promotes tube formation in human coronary artery endothelial cells

Primary tube formation was observed in Exendin-4 treatment group and DDP-4 silence group (fig. 4A), while combination of Exendin-4 and DDP-4 silence induced more intact tubes. In addition, compared with other groups, combination of Exendin-4 and DDP-4 silence had the most numbers of capillary-like tubes , suggesting combination promoted angiogenesis (fig. 4B).

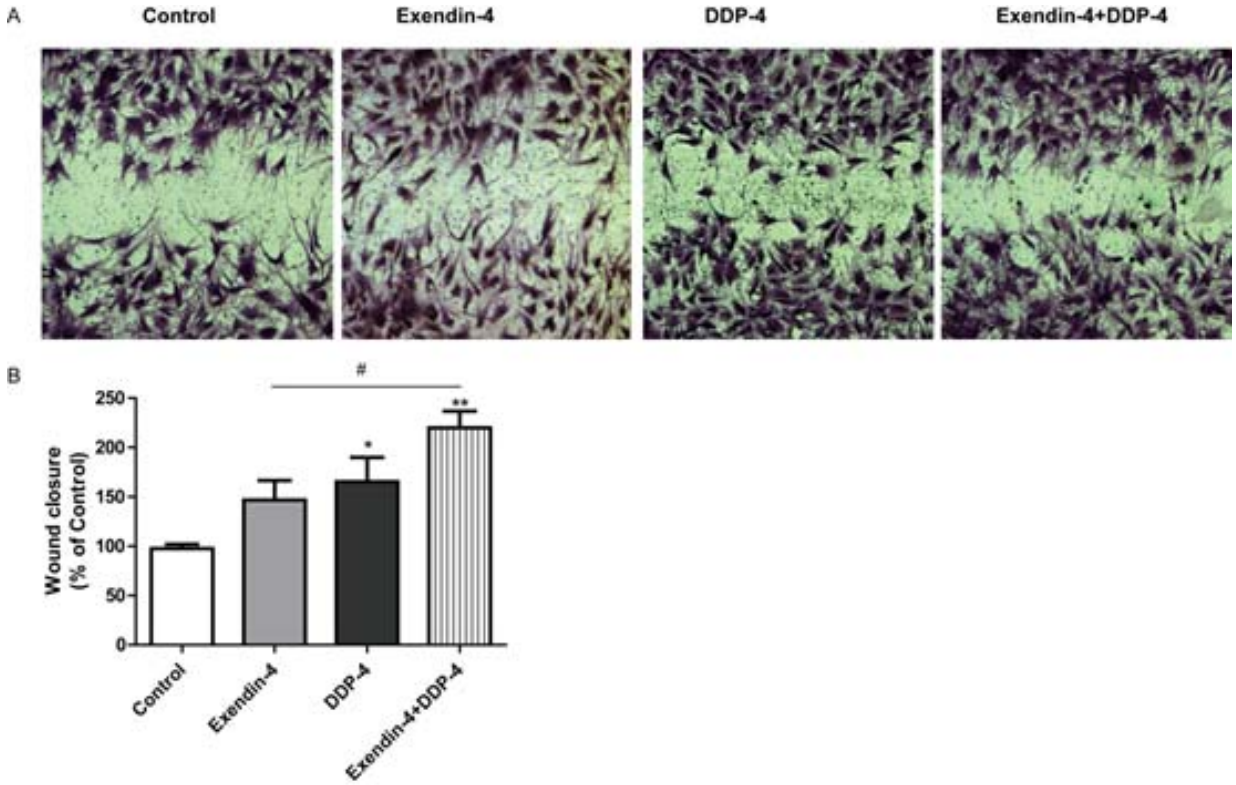

Fig. 3: Effects of Exendin-4 and DDP-4 on wound healing. After pretreatment with exendin-4, DDP-4 siRNA or exendin-4 plus DDP-4 siRNA, the effects on HCAECs wound healing was evaluated by scratch assay. (A) Image of scratch assay; (B) Wound closure rate. * $p<0.05$, ** $p<0.01$ compared to the control group. $^{\#}$ $p<0.05$ compared to the Exendin-4 group.

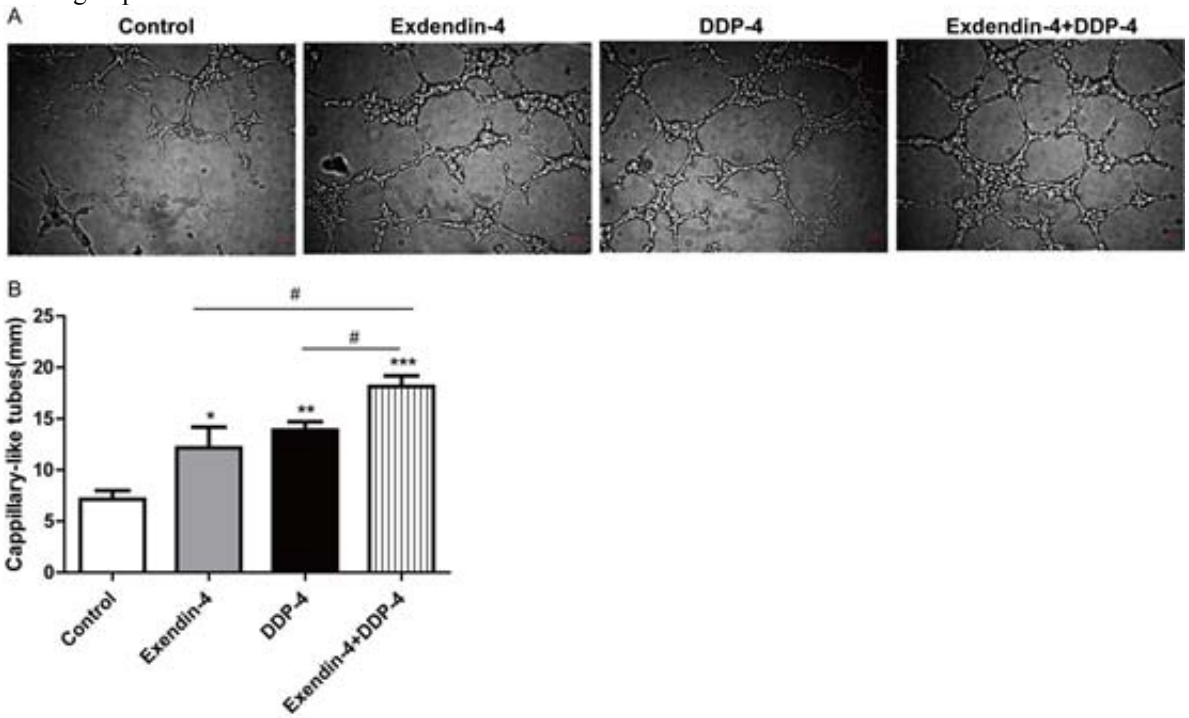

Fig. 4: Effects of Exendin-4 and DDP-4 on tube formation. After pretreatment with exendin-4, DDP-4 siRNA or exendin-4 plus DDP-4 siRNA, the effects on tube formation was determined using Matrigel assay. (A) Picture of in vitro tube formation; (B) Capillary-like tubes. *$p<0.05$, **$p<0.01$ compared to the control group. $^{\#}p<0.05$ compared to the Exendin-4 or DDP-4 group.

Exendin-4 and DDP-4 silence inhibits apoptosis in human coronary artery endothelial cells

Both Exendin-4 treatment and DDP-4 silence indeed decreased the percentage of apoptosis in HCAEC, verified by TUNEL staining (fig. 5A). Combination of Exendin-4 and DDP-4 silence significantly decreased apoptosis (fig. 5B).

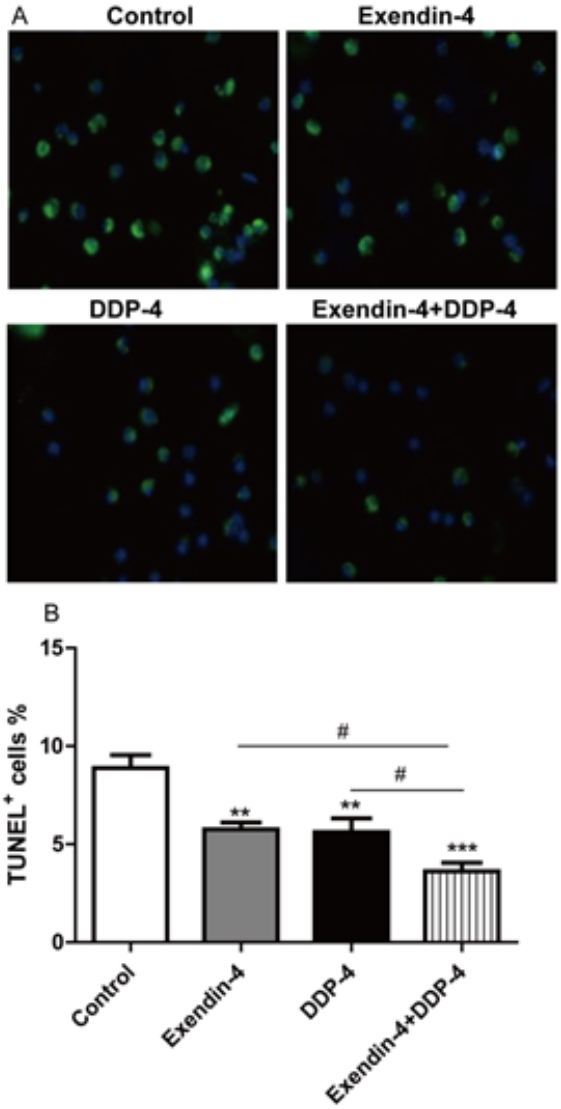

Fig. 5: Effects of Exendin-4 and DDP-4 on apoptosis of HCAECs. After pretreatment with exendin-4, DDP-4 siRNA or exendin-4 plus DDP-4 siRNA, effects on apoptosis of HCAECs was tested by TUNEL staining. (A) Picture of in vitro TUNEL staining; (B) Percentages of TUNEL positive cells. $^{**}p<0.01$, $^{***}p<0.001$ compared to the control group. $^{\#}p<0.05$ compared to the Exendin-4 or DDP-4 group.

Exendin-4 and DDP-4 silence activates PI3K/Akt pathway

To investigate potential mechanisms, we performed Western blotting of PI3K/Akt pathway, including their phosphorylation form. Exendin-4 treatment did not change expression of PI3K and Akt, but increased their phosphorylation levels (p-PI3K, p-Akt, fig. 6). The same phenomenon was observed in DDP-4 silence. Compared with other groups, combination of Exendin-4 and DDP-4 silence also did not influence PI3K and Akt, but significantly increased their phosphorylation levels (fig. 5B).

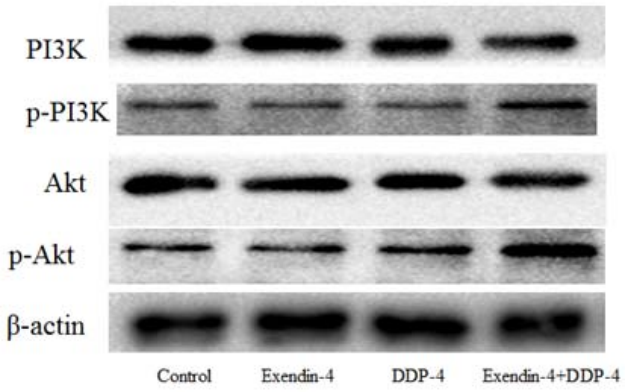

Fig. 6: Effects of Exendin-4 and DDP-4 on PI3K/Akt pathway.

DISCUSSION

In the present study, DDP-4 silence and exendin-4 indeed separately promoted angiogenesis *in vitro*, including migration, invasion and tube formation. What's more, we proved that combination of DDP-4 silence and exendin-4 further enhanced such angiogenesis via activating PI3K/Akt pathways to inhibit apoptosis.

Pathological neovascularization is associated with poor prognosis of atherosclerosis, in which angiogenesis is involved (Panjwani *et al*., 2013). Previous study has showed that aberrant angiogenesis was risk factor for plaque vulnerability (Pescetelli *et al*., 2015). Moreover, adequate regulation of angiogenesis stabilized atherosclerosis plaque (Rask-Madsen and King, 2007). In another hand, although DDP-4 silence and exendin-4 were reported to have promising effect on prognosis of diabetes, clinical cases have indicated that improper or overdose treatment could cause pathological neovascularization, which instead worsen prognosis (Saboo *et al*., 2016; Wajchenberg, 2007).

Both DDP-4 silence and exendin-4 have been proved to be a potential risk factor for atherosclerosis (Wang *et al*., 2016), while their combined effect remains unclear. In our study, DDP-4 silence and exendin-4 indeed separately promoted angiogenesis via multiple mechanisms, including enhancing proliferation, promoting migration, activating invasion and reducing apoptosis. Moreover, we firstly proved that combination of DDP-4 silence and exendin-4 further promoted angiogenesis. This suggested that patients with atherosclerosis should avoid

combination of DDP-4 inhibitor and exendin-4, especially the diabetes patients complicated by atherosclerosis. Moreover, such combination might be used for patients who need angiogenesis for better prognosis, such as ischemic stroke and myocardial infarction.

We further explore mechanisms underlying promoting effect of combined treatments. Combination of DDP-4 silence and exendin-4 did not significantly reduce expressions of PI3K and Akt, but increased their phosphorylation level, verified by Western blotting. These findings indicated that combination of DDP-4 silence and exendin-4 activated PI3K/Akt so as to trigger down-stream apoptosis. Intriguingly, there was also report that pro-apoptosis effect on angiogenesis could improve prognosis of patients with atherosclerosis.

CONCLUSION

In conclusion, we proved that combination of DDP-4 silence and exendin-4 had additional promoted effects on angiogenesis via reducing apoptosis. This was a risk for patients with atherosclerosis, and should be emphasized in clinical scenario.

REFERENCES

Buldak L, Machnik G, Buldak RJ, Labuzek K, Boldys A, Belowski D, Basiak M and Okopien B (2016). Exenatide (a GLP-1 agonist) expresses anti-inflammatory properties in cultured human monocytes/macrophages in a protein kinase A and B/Akt manner. *Pharmacological Reports PR.,* **68**: 329-337.

Calles-Escandon J and Cipolla M (2001). Diabetes and endothelial dysfunction: A clinical perspective. *Endocrine Reviews* **22**: 36-52.

Carmeliet P (2003). Angiogenesis in health and disease. *Nature Medicine,* **9**: 653-660.

Cheng C, Chrifi I, Pasterkamp G and Duckers HJ (2013). Biological mechanisms of microvessel formation in advanced atherosclerosis: The big five. *Trends in Cardiovascular Medicine,* **23**: 153-164.

Chistiakov DA, Orekhov AN and Bobryshev YV (2015). Contribution of neovascularization and intraplaque haemorrhage to atherosclerotic plaque progression and instability. *Acta Physiologica,* **213**: 539-553.

de Vries MR and Quax PH (2016). Plaque angiogenesis and its relation to inflammation and atherosclerotic plaque destabilization. *Current Opinion in Lipidology*,

Di Stefano R, Felice F and Balbarini A (2009). Angiogenesis as risk factor for plaque vulnerability. *Current Pharmaceutical Design,* **15**: 1095-1106.

Drucker DJ and Nauck MA (2006). The incretin system: Glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet,* **368**: 1696-1705.

Erdogdu O, Eriksson L, Nystrom T, Sjoholm A and Zhang Q (2012). Exendin-4 restores glucolipotoxicity-induced gene expression in human coronary artery endothelial cells. *Biochemical and Biophysical Research Communications* **419**: 790-795.

Hotamisligil GS (2010). Endoplasmic reticulum stress and atherosclerosis. *Nature Medicine,* **16**: 396-399.

Jeziorska M and Woolley DE (1999). Neovascularization in early atherosclerotic lesions of human carotid arteries: Its potential contribution to plaque development. *Human pathology,* **30**: 919-925.

Kawasaki K, Smith RS, Jr., Hsieh CM, Sun J, Chao J and Liao JK (2003). Activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway mediates nitric oxide-induced endothelial cell migration and angiogenesis. *Molecular and Cellular Biology* **23**: 5726-5737.

Li Y, Zhu Y, Deng Y, Liu Y, Mao Y, Wang J and Sun J (2016). The therapeutic effect of bevacizumab on plaque neovascularization in a rabbit model of atherosclerosis during contrast-enhanced ultrasonography. *Scientific Reports,* **6**: 30417.

Moreno PR, Purushothaman M and Purushothaman KR (2012). Plaque neovascularization: Defense mechanisms, betrayal, or a war in progress. *Annals of the New York Academy of Sciences,* **1254**: 7-17.

Panjwani N, Mulvihill EE, Longuet C, Yusta B, Campbell JE, Brown TJ, Streutker C, Holland D, Cao X, Baggio LL and Drucker DJ (2013). GLP-1 receptor activation indirectly reduces hepatic lipid accumulation but does not attenuate development of atherosclerosis in diabetic male ApoE(-/-) mice. *Endocrinology,* **154**: 127-139.

Pescetelli I, Zimarino M, Ghirarduzzi A and De Caterina R (2015) Localizing factors in atherosclerosis. *Journal of Cardiovascular Medicine,* **16**: 824-830.

Rask-Madsen C and King GL (2007). Mechanisms of Disease: endothelial dysfunction in insulin resistance and diabetes. *Nature clinical practice Endocrinology & Metabolism,* **3**: 46-56.

Saboo A, Rathnayake A, Vangaveti VN and Malabu UH (2016). Wound healing effects of dipeptidyl peptidase-4 inhibitors: An emerging concept in management of diabetic foot ulcer-A review. *Diabetes & Metabolic Syndrome* **10**: 113-119.

Wajchenberg BL (2007). beta-cell failure in diabetes and preservation by clinical treatment. *Endocrine Reviews,* **28**: 187-218.

Wang Y, Qiu J, Luo S, Xie X, Zheng Y, Zhang K, Ye Z, Liu W, Gregersen H and Wang G (2016). High shear stress induces atherosclerotic vulnerable plaque formation through angiogenesis. *Regenerative Biomaterials,* **3**: 257-267.