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Gemigliptin, a potent selective dipeptidyl peptidase 4 inhibitor, protects endothelial progenitor cells by oxidative stress via caspase-3 dependent pathway

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ABSTRACT

Endothelial progenitor cells (EPCs) are exclusive players in vasculogenesis and endothelial regeneration. EPCs are of two types and their differentiation is mediated by different growth factors. A decrease in EPC number and function causes cardiovascular abnormalities and reduced angiogenesis. Various studies has documented a role of EPCs in diabetes. EPCs treatment with different drugs improve insulin secretion but causes other abnormalities. In vivo and in vitro studies have reported anti glycation effect of gemigliptin but no data is available on in vitro effect of gemigliptin on EPC number and functional credibility. The current study was aimed to find an in vitro effect of gemigliptin on EPC number and function along with an effective treatment dose of gemigliptin. EPCs were isolated, cultured and phenotypically characterized using Dil- AcLDL and ulex-lectin fluorescence staining. EPCs were then treated with different doses of Zemiglo and their viability analyzed with viability assay using water-soluble tetrazolium salt (WST-1), by Annexin V and Propidium Iodide (PI) staining, senescence-associated beta-galactosidase (SA-β-gal) staining, western blot and Flow cytometric analysis of apoptotic signals. The results demonstrated that the isolated EPCs has typical endothelial phenotypes. And these EPCs were of two types based on morphology i.e., early and late EPCs. Gemigliptin dose dependently improved the EPCs morphology and increased EPCs viability, the most effective dose being the 20 μM. Gemigliptin at 10 μM, 20 μM and 50 μM significantly increased the BCL-2 levels and at 20 μM significantly decreased the Caspase-3 levels in EPCs. In conclusion, gemigliptin dose dependently effects the EPCs viability and morphology through Caspase-3 signaling. Our results are the first report of gemigliptin effect on EPC viability and morphology.

1. Introduction

Endothelial progenitor cells (EPCs) inhabit the bone marrow tissues stem cells as well as the peripheral blood circulation [1] and play pivotal role in new blood vessels formation and the repair of endothelial dysfunction [2,3]. EPCs are of two types both of which are involved in neovascularization [4]. Various growth factors like hepatocyte growth factor, vascular endothelial growth factor (VEGF), granulocyte colony stimulating factor, interleukin-6 (IL-6), and endothelial nitric oxide synthase (eNOS) [5] mediate EPCs movement and differentiation to ischemic region. A decrease in EPC number and function leads to impaired vascularization in coronary artery disease (CAD) patients [6]. The levels of circulating EPCs are an indicator of cardiovascular abnormalities [7]. A reduced paracrine angiogenic activity and an increased senescence of circulating endothelial cells has been reported in migraine and Alzheimer disease (AD) patients [8,9]. EPC promoted wound healing and neovascularization into diabetic mice injected with EPCs conditioned medium [10]. EPCs of Bone marrow origin play role in diabetes [11]. EPCs treated with atorvastatin, KMUP-1(7-(2-(4 (2-chlorophenyl) piperazinyl)ethyl)-1,3-dimethylxanthine), and simvastatin were significantly prevented from the hypoxia induced EPCs death [12].

Type 2 diabetes mellitus (T2DM) is characterized by peripheral tissue insulin resistance and impaired insulin secretion by pancreatic beta-cells which leads to hyperglycaemia. T2DM patients are at an increased risk of heart failure due to hyperglycemia, diabetic cardiomyopathy, microvascular damage and autonomic neuropathy. T2DM is also a risk factor for Alzheimer's and parkinson's. Many available treatments improve insulin secretion but these treatments may cause hyper and

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[1]**Abbreviations**

EPCs	Endothelial progenitor cells
WST-1	Water-soluble tetrazolium salt
PI	Propidium Iodide
SA-β-gal	Senescence-associated beta-galactosidase
VEGF	Vascular endothelial growth factor
IL-6	Interleukin-6
eNOS	Endothelial nitric oxide synthase
CAD	Coronary artery disease
AD	Alzheimer disease
T2DM	Type 2 diabetes mellitus
DPP4	Dipeptidyl peptidase
GIP	Glucose dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
MNC	Mononuclear cell
HBSS	Hank's balanced salt solution
EGM-2	Endothelial growth medium-2
EBM-2	Endothelium basal medium-2
FBS	Fetal bovine serum
hEGF	Human epidermal growth factor
hfGF- β	Human fibroblast growth factor β
IGF-1	Insulin-like growth factor-1
PS	Penicillin-streptomycin
Dil- AcLDL	1,1′-dioctadecyl-3,3,3', 3'-tetramethyindocarbocyanine-labeled acetylated low density lipoprotein
Ulexlectin	Ulex europaeus agglutinin I
H2O2	Hydrogen peroxide
CCK-8	Cell Counting Kit-8
BCA	Bicinchoninic acid assay
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBST	Tris buffered saline containing 0.1% v/v Tween-20
FACS	Fluorescence activated cell sorting

hypoglycaemia [13], weight gain (sulfonylureas and meglitinidies) and gastrointestinal intolerance (metformin and alpha glucosidase inhibitors) [14].

Dipeptidyl peptidase (DPP4) has been associated with several disease pathogenesis such as T2DM, renal diseases and vascular diseases [15]. DPP4 inhibitors stimulate insulin secretion by using glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) receptors. Gemigliptin (LC15-0444, brand name; Zemiglo) tartarate sesquihydrate is a highly selective and competitive DPP4 inhibitor [14]. Gemigliptin at different doses significantly reduced fasting plasma glucose levels and HbA1c in T2D patients. Gemigliptin prevented diabetes induced podocyte apoptosis in diabetic mice model. In vivo and in vitro studies have reported anti glycation effect and cardiovascular protective effect of gemigliptin [16]. Although the studies have reported effect of gemigliptin on glucose levels, cardiovascular damage, and a few of them also observed its effect on blood vessel regeneration but there is no data available on in vitro effect of gemigliptin on EPC number and functional credibility as these are the crucial revascularization indicators. EPC functional activity represents a characteristic difference between early and mature EPCs.

The objective of this study was to find out a possible pleiotrophic effect of gemigliptin on vascular EPCs by examining the level of vascular EPC following gemigliptin treatment. It was also aimed at checking the effective treatment dose of gemigliptin. This was carried out by treating cultured EPCs with different doses of gemigliptin.

2. Materials and methods

2.1. Ethics statement

All experimental protocols were performed in compliance with relevant laws and institutional guidelines and approved by the Institutional ethics committee of Seoul National University Hospital (Approval No. H-1603-118-750). Written informed consent was signed by all male and female participants aged from 50 to 85 years. Our research work is in accordance with the ‘‘World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects’’.

2.2. Data collection and access

Blood samples were collected from participants from 2022.04.01 to 2022.04.06. Blood samples were accessed for research purposes at 2022.04.21.

2.3. EPCs isolation and culture

Peripheral blood (6 mL) samples were obtained from diabetes mellitus patients. The mononuclear cell (MNCs) isolation and culture method was followed as described previously with necessary modifications [8,17]. MNCs were fractioned from other components of peripheral blood by centrifugation on Histopaque 1077 (Sigma–Aldrich, Gillingham, Dorset, UK) gradients according to the manufacturer's instructions. Briefly, 6 mL of Histopaque-1077 was centrifuged with 6 mL Peripheral blood sample at 400g for 30 min at room temperature. The mononuclear fraction was separated and mixed with Hank's balanced salt solution (HBSS, 10 mL; Invitrogen, Inchinnan Business Park, Paisley, UK) and centrifuged at 250 g for 10 min at room temperature. Isolated EPCs were re-suspended in the endothelial growth medium-2 (EGM-2) Bullet Kit system (catalog number CC-3162; Lonza, Clonetic, USA) consisting of Endothelium basal medium-2 (EBM-2), 5 % fetal bovine serum (FBS), human epidermal growth factor (hEGF), vascular endothelial growth factor (VEGF), human fibroblast growth factor (hfGF- β), insulin-like growth factor-1 (IGF-1), 1% penicillin-streptomycin (PS) ascorbic acid and heparin. 1x 107 EPCs were seeded on 1% gelatin (Sigma-Aldrich, USA) coated culture plates in (EGM-2) and incubated in a 5% CO2 incubator at 37 °C. Under daily observation, first media change was performed seven days after plating. Thereafter, media was changed daily. EPCs were cultured for further use.

2.4. Phenotype Characterization of EPCs: Dil- AcLDL and ulexlectin uptake

To confirm the EPC phenotype, EPC cells were incubated with 1,1′-dioctadecyl-3,3,3', 3'-tetramethyindocarbocyanine-labeled acetylated low density lipoprotein (Dil- AcLDL) (Dil- AcLDL, cat no. L3484; Thermofischer Scientific. CA, USA) (10 μg/mL) for 1 h at 37 °C, 5 % CO2 and then incubated with FITC-labeled Ulex europaeus agglutinin I (ulexlectin) (FITC labeled Ulex europaeus agglutinin (UEA)-1, cat no. L9006-1 MG; Sigma) (10 μg/mL) for 4 h at room temperature. Cells were subsequently fixed with 4% paraformaldehyde.

2.5. EPCs treatment with gemigliptin

To measure an effect of gemigliptin and evaluation of characteristic effective dose, EPCs were incubated with different doses of gemigliptin (LG Life Science Ltd., Seoul, South Korea) at concentrations of 10 μM, 20 μM, 50 μM, and 100 μM for 48 h. Media was changed every two days. Untreated EPCs comprised the control group (0 μM). In order to observe the gemigliptin toxicity to EPC, EPCs were treated with Hydrogen

peroxide (H2O2) (300 μM0 μM) for 3 h before gemigliptin treatment.

2.6. Cell viability assay

As the number of EPCs is a characteristic indicator of vascular pathologic state, therefore it was analyzed if gemigliptin has any positive effect on cell viability at any dose treatment. Cell viability was measured by cell proliferation by using Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions. Cells were seeded in a 96-well plate and treated with H2O2 (300 μM0 μM) for 3 h before treatment with gemigliptin, EPCs were then incubated with gemigliptin. After gemigliptin treatment, 10 μl water-soluble tetrazolium salt (WST-1) solution containing 100 μl medium was added to each well, and the cells were incubated at 37 °C and 5% CO2 for 30 min, 1, 2, or 4 h. Absorbance was measured using a plate reader at 450 nm (reference 650 nm).

2.7. Senescence-associated beta-galactosidase (SA-β-gal) staining

In cultured endothelial cells, Senescence-associated beta-galactosidase (SA-β-gal) staining determines the senescence condition [18]. To measure SA-β-gal activity, EPCs were seeded in 6-well Nunc plates coated with 1% gelatin and treated with different doses of gemigliptin (10 μM, 20 μM, 50 μM, and 100 μM). After media removal, cells were stained with the x-Gal staining kit (Senescence-associated beta-galactosidase Staining Kit, cat no. 9860; Cell Signaling Technology) according to the manufacturer's protocol to determine the number of senescent cells. EPCs were then photographed under five random microscopic fields using an optical microscope (OLYMPUS, Tokyo, Japan).

2.8. Western blot analysis

EPCs were seeded in a 6-well plate, treated with H2O2 (300 μM0 μM) for 3 h before treatment with gemigliptin, and then incubated with gemigliptin. Cell pellet was obtained by trypsinization and proteins were purified from the pellet by adding RIPA buffer (Radio ImmunoPrecipitation Assay Buffer, Thermo Scientific, Waltham, MA, USA) containing protease inhibitor and phosphatase inhibitor (Roche, NJ, USA). Extracted cellular protein content was measured using a Bicinchoninic acid assay (BCA) protein assay kit (Pierce, Rockford, IL, USA). 15 μg of protein sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 15% gel) at 30 mA for about 2 to 3 h, transferred to nitrocellulose membrane at 100v for 1h. Membrane was blocked in 5% nonfat dry milk dissolved in 1% TBST (Tris buffered saline containing 0.1% v/v Tween-20) for 1 h at room temperature. Membrane was then incubated with primary antibody overnight at 4 °C with. Primary antibodies used are as follows: anti-P53 (1:200; Santa Cruz, CA, USA), anti-Bax (1:200; Santa Cruz, CA, USA), anti-Bcl-2 (1:200; Santa Cruz, CA, USA), cleaved caspase 3 (1:1000; Cell signaling, Beverly, MA, USA), and anti-β-actin (1:200; Santa Cruz, CA, USA). Membrane was then incubated with secondary antibodies for 1 h at room temperature. Secondary antibodies used are as follows: horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies (1:3000, GE Healthcare, NJ, USA). Membrane was then treated with ECL solution (Enhanced chemiluminescence solution, Advansta, CA, USA). Band intensities were measured from three independent observations normalized by β-actin using ImageJ software.

2.9. Flow cytometric analysis: annexin V and Propidium Iodide (PI) staining: cell apoptosis

1 x 10^6 EPCs were suspended in 1 ml cold binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2). 1 x 10^5 cells were incubated with 10 μl of annexin V-FITC (BD Biosciences Pharmingen, San Jose, CA, Cat.no. 556420) for 30 min at room temperature and then incubated with 2 μg/ml of PI (BD Biosciences Pharmingen, Westwood, MA, Cat.no. 556463) for 10 min at room temperature. After incubation, 400 μl of binding buffer was added and fluorescence activated cell sorting (FACS) was performed (FACS Calibur, BD Bioscience, CA, USA). FITC and PI fluorescence were passed through 520 and 630 nm bandpass filters, respectively, and data were analyzed using Flowing Software (www.flowingsoftware.com). The percentage of apoptotic cells was measured by FACS (BD Accuri C6), live cells (FITC-annexin V-/PI-), apoptotic cells (FITC-annexin V+/PI-), and dead cells (FITC-annexin V+/PI+).

2.10. Statistical analysis

All data are expressed as means ± standard error of the mean (SEM) and experiments were repeated at least three times. Statistical analysis was performed with Graphpad Prism software (GraphPad Software). All statistical analysis were performed by one-way analysis of variance (ANOVA) with Tukey's post hoc test. P value of < 0.05 was taken as significant.

3. Results

3.1. Isolation and Phenotype Characterization of vascular EPCs: Dil-Ac LDL uptake and ulexlectin binding

EPCs were isolated and cultured by centrifugation on Histopaque 1077 according to manufacturer's instructions to monitor the early and late growth EPCs. It was observed that early growth EPCs (Early growth EPC, Fig. 1A (a, b) proliferate at the beginning of culture (Fig. 1A (1c), but the proliferation stops and degenerates over time. It was demonstrated that late growth EPCs (Late growth EPC, Fig. 1A (1d) proliferate in the late stage of culture and maintains its proliferative capacity even during subculture.

EPC phenotypically express Dil-Ac LDL and ulexlectin and so the cells were stained with both. Cells were visualized with an inverted fluorescent microscope and cells staining positive for both FITC-ulex-lectin (green) and Dil-acLDL (red) were confirmed to be EPCs. Cell nuclei were stained with DAPI (blue) which verified that all the adherent cells were FITC-ulex-lectin (+) and Dil-acLDL (+) and proliferated well (Fig. 1B)

3.2. Gemigliptin exerts a prominent dose dependent modification effect on the EPCs morphology

Our results demonstrated a dose dependent effect of gemigliptin on EPCs morphology. EPCs treated with 10 μM, 20 μM, and 50 μM (Fig. 2A (b, c and d respectively) showed prominent changes in cell morphology in comparison with control group (0 μM) (Fig. 2A (a) after 48 h gemigliptin treatment. No change in EPC morphology was observed in the 100 μM gemigliptin treated EPCs as compared to the control group (Fig. 2A (e). Results shown represent the average of four independent experiments. Viability Assay results confirmed that EPCs viability increased at all doses of gemigliptin treatment as compared to the control group (0 μM) (Fig. 2B). Gemigliptin significantly improved the viability at 20 μM (Fig. 2B). Though gemigliptin dose dependently effect the EPC morphology but gemigliptin increased the cell viability in all experimental groups. It can be said that gemigliptin has an effect on cell viability improvement.

3.3. Senescence-associated beta-galactosidase (SA-β-gal) staining

EPCs were successfully stained with SA-β-gal (Fig. 3A) and it was demonstrated that there was a difference in the degree of EPC staining depending on the concentration level of the gemigliptin. SA-β-gal staining results showed that the EPCs treated with low dose gemigliptin (10 μM, 20 μM, 50 μM) show significantly less staining as compared to the EPC control group (0 μM). However, it was demonstrated that there

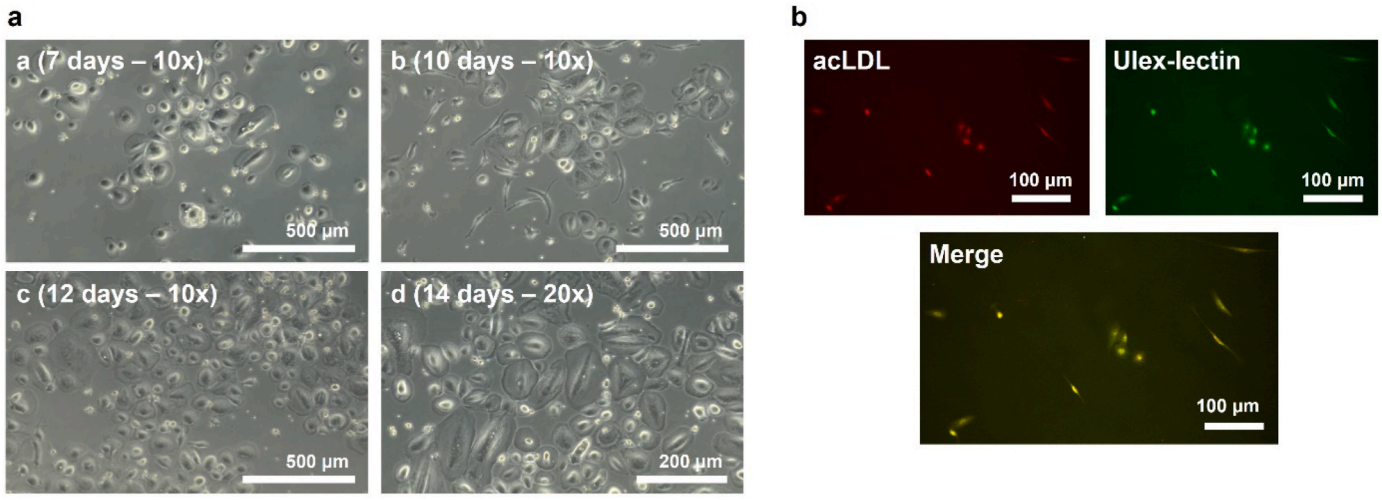

Fig. 1. Morphology and Phenotype Characterization of EPCs. (a) Phase contrast images obtained after inverted microscopy represent two types of cells. The images with 7 (a), 10 (b) and 12 (c) days show Early EPCs which are spindle shaped and efficiently dividing. The 14 days (d) photomicrograph shows late EPCs with cobblestone appearance and are proliferative. **(b)** EPCs were double stained with Dil-Ac LDL (red) and ulex-lectin (green) to validate their endothelial phenotype and the results were observed under a fluorescence microscope. The figure shows that EPCs are stained with Dil-acLDL (red) as well with FITC-ulex-lectin (Green). EPCs were positively stained for both FITC-ulex-lectin and Dil-acLDL which confirmed their endothelial phenotype. Cell nuclei were stained with DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

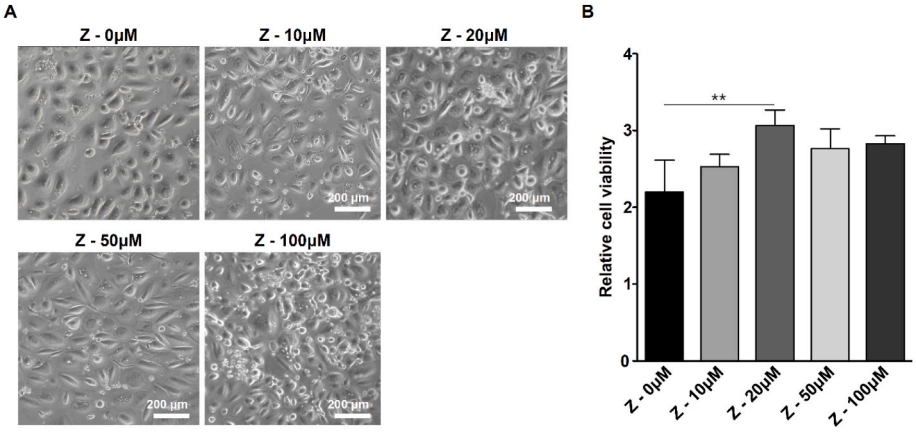

Fig. 2. Gemigliptin dose dependently modifies the EPCs morphology and increases the EPCs viability. (a) The photomicrographs show the effect of gemigliptin on EPCs culture at four doses i.e., Z-10 µM (b), Z-20 µM (c), Z-50 µM (d) and Z-100 µM (e). These results indicated a dose dependent effect of gemigliptin on EPCs morphology. A control EPC group (0 µM) (a) was not treated with gemigliptin. EPCs treated with 10 µM, 20 µM, and 50 µM showed prominent changes in cell morphology in comparison with control group (0 µM). EPCs treated with 100 µM gemigliptin show no change in EPC morphology as compared to the control group. **(b)** The bar graphs represent the effect of four independent doses of gemigliptin Z-10 µM, Z-20 µM, Z-50 µM, Z-100 µM in a viability assay on cell viability through cell proliferation. The results indicated that gemigliptin improved viability in all EPCs groups when compared with control group. The highest viability being observed at 20 µM.

was no staining difference in the EPCs treated with high dose gemigliptin (100 µM) as compared to the EPC control group (Fig. 3B).

3.4. Gemigliptin reduces the apoptotic signals in EPCs; western blot and flow cytometric analysis

We investigated which gemigliptin dose activated or inhibited the apoptotic pathway. Phosphorylation of P53, BAX, BCL-2 and Caspase-3 was measured by western blot (Fig. 4 A). Whole uncropped images of the original western blots are demonstrated in Supporting information (Figs. S1–S5). We demonstrated that gemigliptin at 10 µM, 20 µM and 50 µM significantly (where * is $P \leq 0.05$ and ** is $P \leq 0.01$) increased the BCL-2 levels (Fig. 4 A, B) in EPCs as compared to the control EPC group (0 µM gemigliptin). Gemigliptin at 20 µM significantly (where * is $P \leq 0.05$ and ** is $P \leq 0.01$) increased the BCL-2 levels in EPCs as compared to the control EPC group (0 µM gemigliptin) and all other doses. Interestingly, gemigliptin at 20 µM significantly (where * is $P \leq 0.05$ and ** is $P \leq 0.01$) decreased the Caspase-3 levels in EPCs as compared to the control EPC group (0 µM gemigliptin). P53 and BAX showed no significance change at any dose of gemigliptin. At 50 µM, gemigliptin reduced the Caspase-3 expression as compared to control (0 µM) (Fig. 4B). A strong effect of other gemigliptin dose treatments was not demonstrated in EPCs.

To demonstrate the possible pleiotropic effects of gemigliptin, FACS was performed to determine cell viability of EPCs treated with different doses of gemigliptin. Fluorescence activated cell sorting (FACS Calibur, BD Bioscience, CA, USA) was performed to monitor annexin V and PI fluorescence. FITC annexin V and PI fluorescence were passed through 520 and 630 nm bandpass filters, respectively, and data were analyzed using Flowing Software (www.flowingsoftware.com). The percentage of apoptotic cells was measured by FACS (BD Accuri C6), live cells (FITC-annexin V-/PI-), apoptotic cells (FITC-annexin V+/PI-), and dead cells

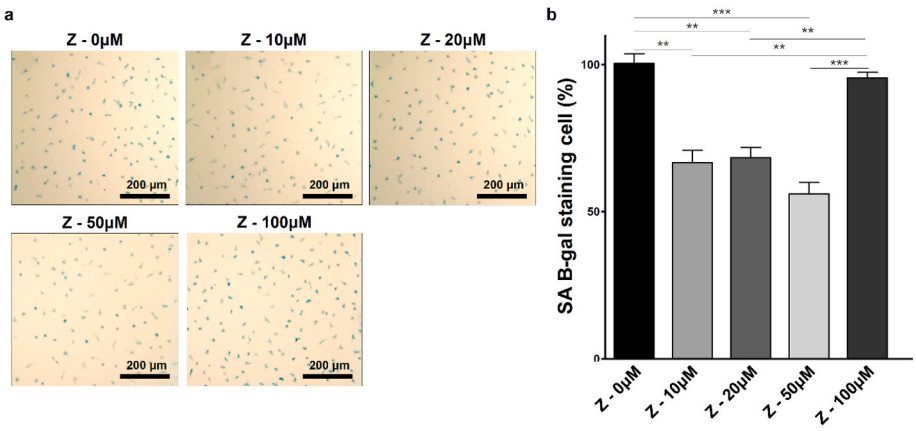

Fig. 3. Senescence-associated beta-galactosidase (SA-β-gal) Staining. (a) EPCs were treated with four doses of gemigliptin and then were stained with Beta galactosidae. Photomicrographs show that EPCs were successfully stained with SA-β-gal at all doses. **(b)** The bar graphs demonstrated that EPCs treated with low dose gemigliptin (10 μM, 20 μM, 50 μM) show significantly less staining as compared to the EPC control group (0 μM). However, it was demonstrated that there was no staining difference in the EPCs treated with high dose gemigliptin (100 μM) as compared to the EPC control group (0 μM).

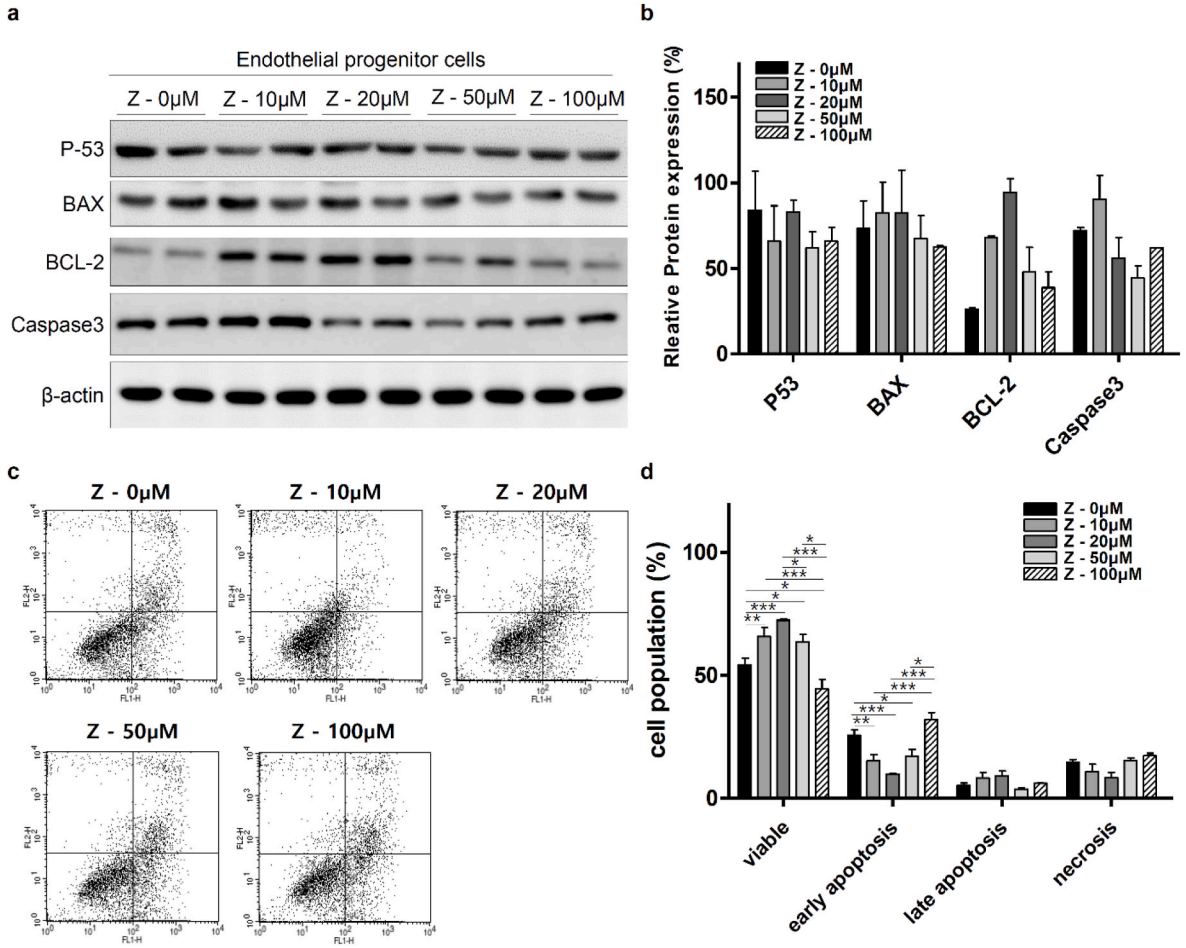

Fig. 4. Gemigliptin reduces the apoptotic signals in EPCs. (a) Western Blot. Phosphorylation of P53, BAX, BCL-2 and Caspase-3 was measured by western blot. The membrane image shows the expression of P53, BAX, BCL-2 and Caspase-3 at all doses of gemigliptin. **(b)** The bar graph shows that gemigliptin at 10 μM0 μM, 20 μM0 μM and 50 μM significantly (where * is $P \leq 0.05$ and ** is $P \leq 0.01$) increased the BCL-2 levels in EPCs as compared to the control EPC group (0 μM0 μM gemigliptin). Gemigliptin at 20 μM0 μM significantly (where * is $P \leq 0.05$ and ** is $P \leq 0.01$) increased the BCL-2 levels in EPCs as compared to the control EPC group (0 μM gemigliptin) and all other doses. Gemigliptin at 20 μM significantly (where * is $P \leq 0.05$ and ** is $P \leq 0.01$) decreased the Caspase-3 levels in EPCs as compared to the control EPC group (0 μM gemigliptin). P53 and BAX showed no significance change at any dose of gemigliptin. At 50 μM, gemigliptin reduced the Caspase-3 expression as compared to control (0 μM). **(c)** Flow cytometric analysis revealed the percentage of live cells (FITC-annexin V-/PI-), apoptotic cells (FITC-annexin V+/PI-) and dead cells (FITC-annexin V+/PI+). **(d)** Bar photomicrographs show that gemigliptin at 10 μM, 20 μM and 50 μM doses significantly increased the viability of EPCs and significantly decreased the early apoptosis at the same doses.

(FITC-annexin V+/PI+) (Fig. 4C). Gemigliptin at 10 μM, 20 μM and 50 μM doses significantly increased the viability of EPCs and significantly decreased the early apoptosis at the same doses. Our FACS results analysis were demonstrated to be in line with our Western results (Fig. 4D).

4. Discussion

Our study first time provides the evidence of an effect of gemigliptin on EPCs proliferation capacity and morphological characteristics. Moreover, a dose dependent role of gemigliptin on EPC viability and proliferation was also demonstrated.

Bone marrow [19], human umbilical vein [20], peripheral blood circulation [21] and are some of the sources from which EPCs have been isolated using density gradient centrifugation. Two types of EPCs have been isolated from peripheral blood which are called early (spindle like cells with low proliferation capacity) and late EPCs (cobblestone-shaped cells which increase in number) [17]. EPCs role in neovasculogenesis has been shown by Pyšná et al. [22], and they also demonstrated that EPC counts were significantly lower in people with diabetes. We successfully isolated and cultured EPCs from which two types of EPCs (early and late EPCs) were observed. Our results showed that early EPCs increase in number at the beginning of culture, however these EPCs lost their proliferation activity after a few days in culture. Late growth EPCs divide later in the culture and continue to divide in subsequent subcultures as well.

Uptake of Dil Ac LDL and UEA-1 lectin binding are important EPC identification markers [23]. EPCs in our experimental set up of Dil Ac LDL and UEA-1 lectin fluorescence staining were also demonstrated to be fluorescently double stained with Dil Ac LDL and UEA-1 lectin as observed under a fluorescence microscope.

EPCs number is a predictor of the cardiovascular pathogenesis [24] and is decreased in cardiovascular abnormalities and diabetes [6] and peripheral vascular complications [25]. It has been reported that the EPC number in diabetic patients was significantly lower than that in the normal people. Although the extent of decrement of EPCs varies with studies, the EPC of diabetic patients is reduced compared to normal people by approximately ~40% [38–40]. Gemigliptin at different doses significantly reduced fasting plasma glucose levels and HbA1c in T2D patients and high fat fed mice [26,27]. In vivo and in vitro studies have reported anti glycation effect and cardiovascular protective effect of gemigliptin [16]. Our results, for the first time, revealed a direct dose dependent effect of gemigliptin on EPCs morphology. EPCs treated with 10 μM, 20 μM, and 50 μM showed prominent changes in cell morphology in comparison with control group. In addition, considering the reduced extent of EPCs by diabetes is about ~40%, the effect of gemigliptin is significant from the EPC perspective. All experiments to prove the effect of gemigliptin on the EPCs were conducted in the oxidative stress environment by H_2O_2, to accelerate the experiments and clarify the results.

An induction of various growth factors into EPCs increase differentiation. Induced EPCs secrete growth factors which activate the repair of damaged vascular endothelial cells [3,28]. Bone marrow derived EPCs are mobilized and cause an in vitro increase in differentiated EPCs if induced by VEGF [29], β- Fibroblast growth factors (FGF) and thrombin [30]. IL-6 stimulated EPC proliferation and migration [31]. KMUP-1 (7-(2-(4(2-chlorophenyl) piperazinyl) ethyl)-1,3-dimethylxanthine), atorvastatin and simvastatin treated EPCs significantly prevented the hypoxia induced EPCs death and apoptosis [12].

Our data from viability Assay demonstrated that EPCs viability increased at all doses of gemigliptin treatment as compared to the control group (0 μM). Though gemigliptin dose dependently effect the EPC morphology but gemigliptin increased the cell viability in all experimental groups.

Endothelial cells undergo senescence due to impaired migration from the bone marrow and some molecular mechanisms such as an enhanced caspase-3 which cause apoptosis in EPCs change the differentiation capacity of EPCs due to hyperglycemia [32–34].

It was demonstrated in this study that SA-β-gal staining represented a difference in the degree of EPC staining depending on the concentration level of the gemigliptin. EPCs treated with low dose gemigliptin show staining difference as compared to the EPC control group.

Drugs like metformin [35], sitagliptin [36] and simvastatin [37] use p13k/Akt/eNOS and ROS pathway to improve EPC movement and differentiation to the damaged site. We demonstrated that gemigliptin reduced the cell death signal in EPCs at some doses, when compared with the control EPC group. Gemigliptin at 20 μM concentration improved all signals except P53 in EPCs. Gemigliptin at 20 μM and 50 μM doses were effective in improving EPCs death as shown by the FACS analysis results, which is consistent with cell viability result by EPC counting. However, the dose dependency of gemigliptin on restoring EPC function and count did not have full consistency throughout the results in this study. Gemigliptin at 20 μM doses were most effective in cell viability and apoptosis, but gemigliptin at 50 μM doses were most effective in SA-β-gal staining and Caspase-3 expression. This discrepancy might have occurred because the optimized dose of gemigliptin is between 20 μM and 50 μM, and because EPC and caspase-3 are not the only factors controlling cell viability and senescence.

In conclusion this study revealed a highly positive effect of gemigliptin in improving EPCs morphology, enhancing the cellular viability and differentiation potential. Such an effect could be used in developing further studies where gemigliptin role in EPC can be studied at animal model and thus it may ultimately lead to a fast therapeutic candidate of diabetic caused vascular pathogenesis.

5. Conclusion

We concluded, based on our results, that gemigliptin is effective in improving EPCs number and morphology. Gemigliptin, by enhancing the proliferative activity of EPCs exerts a highly positive effect in improving EPCs viability through reducing apoptotic signals. The most effective doses of EPCs were concluded to be 20 μM and 50 μM. It shows that gemigliptin has very positive effect on angiogenesis and regeneration.

Authorship contribution statement

ML and AT contributed equally to this work and share first authorship. AT and ML designed and conceptualized the study. AT wrote the original manuscript and interpreted data. ML carried out the experimental procedures and performed data analysis. MK approved the final version of manuscript to be submitted. All authors reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Ethics statement

All experimental protocols were performed in compliance with relevant laws and institutional guidelines and approved by the Institutional ethics committee of Seoul National University Hospital (Approval No. H-1603-118-750). Written informed consent was signed by all male and female participants aged from 50 to 85 years. Our research work is in accordance with the "World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects''.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

The authors affirm that human research participants provided informed consent for publication of the paper.

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CRediT authorship contribution statement

Mijung Lee: Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Amna Rashid Tariq:** Writing – original draft, Visualization, Software, Investigation. **Manho Kim:** Writing – review & editing, Supervision, Resources, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101673.

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