A novel dressing seeded with embryonic artery CD133⁺ cells and loaded with the Sirt1 agonist SRT1720 accelerates the healing of diabetic ischemic ulcers

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Abstract. Refractory ischemic ulcers that occur in patients with diabetes present a major clinical challenge. Embryonic artery cluster of differentiation 133^+ cells (EACCs) may promote the healing of diabetic ulcers; however, the high glucose environment in the diabetic ulcers decreases the survival rate of transplanted EACCs and inhibit their biological function. Furthermore, microcirculation in diabetic ischemic ulcers is impaired, which inhibits the beneficial effect of EACCs. In the current study, the Sirt1 agonist SRT1720 was selected as a therapeutic drug and loaded into a dressing composed of PLGA, collagen and silk (PCSS) formed using electrospinning technology. EACCs were seeded onto the PCSS dressing and this was used to treat diabetic ulcers. The results indicated that SRT1720 promotes the proliferation of EACCs, enhances the secretion of vascular endothelial growth factor A, interluekin 8 and basic fibroblast growth factor, and inhibits the secretion of tumor necrosis factor α. Furthermore, SRT1720 promoted the paracrine function of EACCs and promoted the proliferation and migration of human umbilical vein endothelial cells. PCSS induced the steady release of SRT1720 over a 15-day period and PCSS seeded with EACCs (PCSS-EACCs) were transplanted into the diabetic ischemic ulcers of mice with diabetes. The results of these experiments indicated that angiogenesis and the healing of diabetic ischemic ulcers was significantly improved following the transplantation of PCSS-EACCs. Therefore, PCSS-EACCs may be a novel and effective treatment for diabetic ischemic ulcers.

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Abbreviations: EACCs, embryonic artery cluster of differentiation 133^+ cells; VEGFA, vascular endothelial growth factor A; IL-8, interleukin-8; PCS, polylactic acid mixed with collagen protein and silk; PCSS, PCS mixed with SRT1720; STZ, streptozotocin; TNF-α, tumor necrosis factor α; bFGF, basic fibroblast growth factor

Key words: diabetes, ulcers, sirtuin1, embryonic artery cluster of differentiation 133^+ cells, paracrine

Introduction

The treatment of refractory ulcers in the feet of patients with diabetes is a major challenge (1). Patients with diabetic foot ulcers often also experience peripheral vascular lesions that cause ischemia, further worsening the ulcers. Such cases may eventually require amputation or result in the mortality of patients (2). Although platelet derived growth factor gel is effective at treating non-ischemic ulcers, it is ineffective at treating ischemic ulcers (3). Therefore, novel therapeutic strategies to treat life-threatening ischemic diabetic ulcers are urgently required.

Previous studies have indicated that transplantation with embryonic artery cluster of differentiation cluster of differentiation (CD)133^+ cells (EACCs) may promote the healing of diabetic ulcers (4). EACCs release vascular endothelial growth factor A (VEGFA) and interleukin-8 (IL-8), which promote the proliferation, migration and angiogenesis of endothelial cells via a paracrine mechanism (5). However, glucose levels in the ulcer region are high, resulting in the inhibition of EACC viability, function and survival; thus, the pathological environment of the ulcer region may limit the efficacy of EACCs in the treatment of diabetic ulcers (6,7). Therefore, it is important to identify methods to effectively enhance the survival and biological function of EACCs in the ulcer area to improve the treatment of diabetic ulcers.

It has been demonstrated that sirtuin (Sirt) family proteins serve an important role in maintaining cell survival and biological activity. The sirtuin family is a family of highly conserved NAD^+ dependent deacetylases, Sirt1 is the most widely studied sirtuin protein at present and a popular drug design target (8). Sirt1 is able to interact with a variety of signal transduction proteins, induce the deacetylation of histone lysine residues and transcription factors, and regulate neuroprotection, cell senescence, apoptosis, lipid metabolism, insulin secretion, inflammation, oxidative stress response and angiogenesis (9,10). Due to the effect of Sirt1 on biomedical regulation and in order to effectively apply Sirt1 in the treatment of diabetes, cardiovascular disease, metabolic syndrome and aging-associated diseases; several Sirt1 agonists have been identified and studied (11,12). Among various Sirt1 agonists, SRT1720 was revealed to be the most effective at activating Sirt1 (11-14).

Therefore, it has been suggested that Sirt1 may be used to enhance the survival rate and function of EACCs in the

ulcer region. In the current study, poly(lactic-co-glycolic acid) (PLGA), collagen and silk were mixed with SRT1720 to form the composite material PCSS using electrospinning technology and EACCs were seeded onto the PCSS to construct the novel dressing to treat patients with diabetic ulcers.

The current study investigated whether PCSS was able to release SRT1720 slowly over a period of 15 days. Furthermore, it was assessed whether EACCs are able to grow well on the PCSS and whether SRT1720 is able to effectively promote the secretion of vascular endothelial growth factor A (VEGFA), interleukin 8 (IL-8) and basic fibroblast growth factor (bFGF) and inhibit the secretion of tumor necrosis factor α (TNF-α) by EACCs. The results of the current study demonstrated that this novel dressing markedly increased the survival rate of EACCs in diabetic ulcers and promoted angiogenesis, thus promoting the healing of diabetic ulcers. Therefore, the PLGA-SRT1720-EACCs composite dressing assessed in the current study may be used as a novel and effective treatment for diabetic ulcers.

Materials and methods

Cell separation and culture. C57 mice (n=10; 5-8 weeks old; weighing 20±4 g; sex ratio, 1:1) were purchased from the Experimental Animal Center of the Third Military Medical University (Chongqing, China). The mice were housed in the specific-pathogen free environment with a temperature of 24-28˚C, relative humidity of 50-60% and natural light cycle. The mice were given sterilized food, and water with bacitracin (4 g/l) and neomycin (4 g/l) *ad libitum*. All procedures performed in animals were approved by the Animal Care and Use Committee of the Third Military Medical University. The adult healthy mice mated and the vaginal plug was observed at 8:00 in the morning, the day that vaginal plug was identified was recorded as gestational age 0 day. After 15-20 days of pregnancy, the fetal aorta-derived vascular $CD133^+$ cells were obtained from aortas of the mouse embryos following a previously reported protocol (15). Briefly, $1x10^6$ cells were separated from the aorta tissue using EDTA (5 mM), centrifuged at 1,000 x g for 3 min at room temperature and incubated with magnetic microbeads (Miltenyi Biotec, Inc., Cambridge, MA, USA) conjugated to the anti-CD133 antibody (cat. no. ab19898; 1:1,000; Abcam, Cambridge, MA, USA) for 30 min at 4˚C. $CD133^+$ cells were separated using the Quadro MACS™ Separation Unit (Miltenyi Biotec, Inc.). $CD133^+$ cells were then cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. In order to simulate the environment of high blood sugar levels *in vitro*, human umbilical vein endothelial cells (HUVECs), which was purchased from American Type Culture Collection (Manassas, VA, USA), were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 30 mM glucose, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. All cells were maintained at 37˚C in 5% CO_2.

Synthesis of composited material. Briefly, 80% polylactic acid, 10% collagen protein and 10% silk (PCS) were dissolved to prepare a mixed solution; the concentration of PCS was adjusted by adding distilled water and the final concentration was 5%. Subsequently, 100 mM SRT1720 (Shanghai BetterBioChem Co., Ltd., Suzhou, China) was added to the mixture to form PCSS. An FM-1205 electrospinning device was purchased from Beijing Future Material Sci-tech Co., Ltd. (Beijing, China). Under an operating voltage of 220 kV/m, the mixed solution was ejected from the nozzle of the electro spinner and collected. The surface of the biological material was photographed using an S-3400N-II scanning electron microscope (Hitachi, Ltd., Tokyo, Japan).

SRT1720 release detection. The PCSS composite material was dissolved in the PBS at 37˚C for 15 days to determine the release of SRT1720. The release of the sample was detected using a UV-VIS spectrometer (Nicolet Evolution 300; Thermo Fisher Scientific, Inc.) at a wavelength of 428 nm and the release ratio was calculated using Beer's law (16).

Growth of EACCs on composite material. The cell was seeded on composite materials, then cell bioactivity was evaluated; the proliferation and adhesion of the cells were the important indexes in the evaluation of cell bioactivity (17). To measure the growth of EACCs, after three passages, $5x10^5$ EACCs were seeded onto the surface of PCS composite material (PCS-EACCs) or PCSS composite material (PCSS-EACCs) following immersion in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C in 5% CO_2. Cells were then incubated for 72 h and cell growth was determined using a LSM 780 NLO laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany).

Enzyme-linked immunosorbent assay (ELISA). Quantitative analysis of the cytokines secreted by EACCs following different treatments was performed using ELISA. TNF-α (cat. no. PT512; Beyotime Institute of Biotechnology, Beijing, China), VEGFA (cat. no. EK0541; Wuhan Boster Biological Technology, Ltd., Wuhan, China), IL-8 (cat. no. EMC104.48) and bFGF (cat. no. EHC130.48; both Neobioscience Technology Company, Shenzhen, China) ELISA kits were used to determine the concentrations of TNF-α, VEGFA, IL-8 and bFGF according to the manufacturer's protocol.

Cell proliferation. The cell proliferation ratio was detected using the Cell-Light™ EdU DNA Cell Proliferation kit (Guangzhou RiboBio Co., Ltd., Guangzhou, China), following the manufacturer's protocol.

Cell migration assay. Cell migration was measured by performing a wound assay. Briefly, $5x10^6$ HUVECs were seeded in the 6-well plate and cultured in RPMI-1640 medium supplemented with glucose, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin, and incubated overnight at 37˚C in 5% CO_2. A wound was created in each well and the plate was washed 3 times with RPMI 1640. Then 2 ml RPMI 1640 medium with 10% FBS was added into each well; after 24 h, the scar areas of each well were observed and photographed using an Olympus BX50 microscope (Olympus Corporation, Tokyo, Japan; magnification, x200)

and were quantified using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

Cell culture media collection. EACCs (5x10^6) were seeded in 6-well plates, 30 nM glucose was added into DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C in 5% CO_2 and incubated at 37˚C in 5% CO_2 overnight. Following the adherence of the cells to the wells, the cells were treated with dimethyl sulfoxide (0.1%), PCS (50 mM), SRT170 (10 mM) or PCSS (50 mM). After 36 h of incubation, the cell culture media were collected and stored at -20˚C.

Cell invasion assay. Cell invasion was measured using a Transwell assay. Transwell chambers were purchased from Corning, Inc. (Corning, NY, USA). Chambers inserted in the lower chamber were coated with diluted Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). A total of 5x10^5 HUVECs in RPMI 1640 medium were incubated in the upper chamber; the lower chambers were filled with 500 μl RPMI-1640 medium with 10% FBS. Subsequently, the collected cell culture media (100 μl) were added to the lower chambers. Following 12 h incubation, insert membranes were collected. Cells were stained with 0.5% crystal violet at room temperature for 30 min, photographed and counted using an Olympus BX50 microscope (magnification, x200).

Animal experiments. A total of 120 C57 mice (5-8 weeks old; weighing 20±4 g; sex ratio, 1:1) were used in the current study to establish the diabetes model. A total of 100 5-8 week old C57 mice weighing 20±4 g received an intraperitoneal injection of 40 mg/kg streptozotocin (STZ) everyday following 12 h fasting over a period of 5 days to establish the diabetes model. The remaining 20 mice were with injected intraperitoneally with 1 ml PBS and used as controls. After 5 days of continuous STZ injections, fasting blood glucose levels were detected using the ACCU-CHEK Active meter (Roche Applied Science, Rotkreuz, Switzerland). The glucose levels >16.7 mM indicated that the diabetes model was successfully established; the results indicated that all 100 mice were successfully induced as diabetes models and the control mice were normal. Mice with diabetes were anesthetized with 30 mg/kg sodium pentobarbital (10 mg/ml) via intraperitoneal injection. Subsequently, the terminal branches of the femoral artery were ligated and the skin tissue was excised at an area of 6x6 mm at the lateral thigh to establish the ischemic diabetic ulcer. Subsequently, ulcers were covered by EACCs, EACCs grown on the PCS materials (PCS-EACCs), EACCs pretreated with SRT1720 (SRT1720-EACCs), PCSS and EACCs grown on the PCSS materials (PCSS-EACCs; all n=20). C57 mice in the control group (n=20) underwent the same ulcer surgery and the ulcers were left untreated. Breathable medical dressings were used to bandage the wounds.

Histological assessments. Bandages were removed 2 days following treatment. Wound recovery was recorded and photographed using a D90 camera (Nikon Corporation, Tokyo, Japan) on days 3, 7 and 14. Wound areas were measured and calculated using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.) to measure wound recovery. On day 14, the tissues surrounding the wounds were collected, fixed with 4% paraformaldehyde at room temperature for 24 h and embedded in paraffin. Tissues were cut into 5-μm-thick sections, and hematoxylin and eosin (H&E) staining was performed using the Hematoxylin and Eosin Staining Kit (cat. no. C0105; Beyotime Institute of Biotechnology, Beijing, China) according to the manufacturer's protocol, then the results were observed by an Olympus BX50 microscope (magnification, x200). In order to further detect the vascular density, the vascular endothelial cell marker CD31 was selected for immunofluorescence staining. The sections were permeabilized with 0.1% triton X-100 for 15 min at room temperature, then incubated by 100% goat serum (cat. no. C0265; Beyotime Institute of Biotechnology) for 30 min at 37˚C. The sections were then incubated with anti-CD31 antibodies (cat. no. ab28364; 1:300; Abcam) diluted in 100% goat serum at 4˚C overnight, washed 3 times with PBS, incubated with Alexa Fluor® 680-conjugated donkey anti-rabbit IgG antibodies (cat. no. A10043; 1:500; Thermo Fisher Scientific, Inc.) diluted in 100% goat serum at 37˚C for 2 h and washed 3 times with PBS. The images of the sections were observed through a laser scanning confocal microscope (TCS-SP5; Leica Microsystems GmbH; magnification, x400) and then the size of endothelium was assessed.

Statistical analysis. All data are presented as the mean ± standard deviation. The nonparametric Mann-Whitney rank-sum test was used to estimate differences between two samples. Intergroup comparisons were performed to assess differences among >2 groups using one-way analysis of variance followed by Bonferroni's correction. P<0.05 was determined to indicate a statistically significant difference.

Results

The characteristics of PCSS-EACCs. To improve the uniformity of the matrix material, electrospinning technology was used to form the PCS (80% polylactic acid, 10% collagen protein and 10% silk) and PCSS (100 nM SRT172 was added to the PCS solution to form the PCSS). Scanning electron microscopy was used to determine the uniformity of the materials and it was identified that the silk itself and the gaps between the silk were uniform, and that the structure of the material was also uniform (Fig. 1A). The materials were regular and uniform and there was no difference in thickness (Fig. 1B). To detect the growth of EACCs on the PCSS, EACCs were seeded on the PCSS and observed using a scanning electron microscope. The results indicated that EACCs were able to grow well on the PCSS, indicating that PCSS effectively promote the growth and proliferation of EACCs (Fig. 1C). To detect the release ability of PCSS for SRT1720, a release experiment was performed and the results indicated that the PCSS is able to release SRT1720 slowly and steadily over a period of 15 days (Fig. 1D). These results indicate that the materials designed in the current study not only promote the growth of EACCs but may also be used to promote the steady release of SRT1720 over a prolonged period.

SRT1720 promotes the biological function of EACCs. As Sirt1 is closely associated with the biological function of cells, the PCSS used in the current study contained the Sirt1

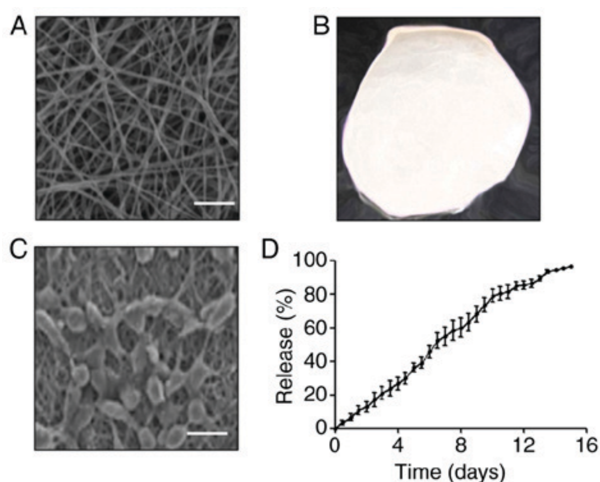

Figure 1. Characteristics of PCSS-EACCs. (A) PCSS was observed using a scanning electron microscope; the surface of PCSS was regular and uniform. Scale bar=10 μm. (B) Biomaterial imaged using a laser scanning microscope. (C) The growth of EACCs on the PCSS was imaged using a scanning electron microscope. Scale bar=100 μm. (D) The release of SRT1720 by PCSS; the release rate was steady and long-lasting. EACCs, embryonic artery cluster of differentiation 133$^+$ cells; PCSS, polylactic acid miced with collagen protein, silk and SRT1720.

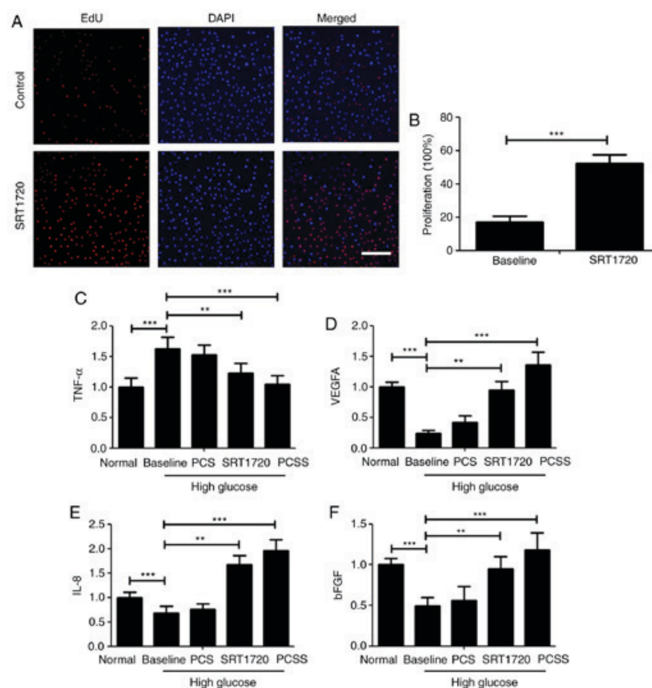

Figure 2. SRT1720 promotes the biological function of EACCs. (A) EACCs were treated with 10 mM SRT1720 and cell proliferation was detected using an EdU kit. Scale bar=200 μm. (B) Quantification of cell proliferation rates. In the normal group, EACCs were cultured in normal medium and this medium was collected for further detection; in the baseline group, EACCs were cultured in high glucose medium and the conditional medium was collected for further detection; EACCs were also co-cultured in high glucose medium and PCS, SRT1720 or PCSS, and the conditional medium was collected for further detection. Enzyme-linked immunosorbent assays were conducted on conditional media from all groups to measure. (C) TNF-α, (D) VEGFA, (E) IL-8 and (F) bFGF levels. $^{**}P<0.01$ and $^{***}P<0.005$. EACCs, embryonic artery cluster of differentiation 133$^+$ cells; VEGFA, vascular endothelial growth factor A; IL-8, interleukin-8; PCS, polylactic acid mixed with collagen protein and silk; PCSS, PCS mixed with SRT1720; TNF-α, tumor necrosis factor α; bFGF, basic fibroblast growth factor.

agonist SRT1720. A number of experiments were conducted to determine the effect of SRT1720 on the biological function of EACCs. To detect cell proliferation, an EdU kit was used. The results demonstrated that SRT1720 significantly promotes the proliferation of EACCs in a high glucose environment (Fig. 2A and B). ELISA experiments were also performed to determine the effects of SRT1720 and PCSS on EACCs in a high glucose environment. TNF-α is an important cytokine that causes cell death (18). In a high glucose environment, the secretion of TNF-α by EACCs increased significantly; however, the secretion of TNF-α by EACCs was significantly decreased following treatment with SRT1720 and PCSS (Fig. 2C). VEGFA induces angiogenesis and promotes cell migration (19). In a high glucose environment, the secretion of VEGFA by EACCs was significantly inhibited; however, treatment with SRT1720 and PCSS significantly increased the secretion of VEGFA by EACCs (Fig. 2D). IL-8 is a multifunctional factor; it is able to stimulate the migration of neutrophils into inflammatory tissue and activate inflammatory cells and is also able to promote fibroblast proliferation. Additionally, IL-8 is a chemotactic cytokine that can promote inflammatory cell chemotaxis and induce cell proliferation (20). The main role of IL-8 is to attract and activate neutrophils, promote the lysosomal enzyme activity of neutrophils and phagocytosis, and have chemotactic effect on basophils and T cells; IL-8 can induce endothelial cell migration and proliferation, further promoting vascular proliferation (20). As angiogenesis facilitates the healing of diabetic ulcers, levels of IL-8 secreted by EACCs in a high glucose environment were measured. The secretion of IL-8 by EACCs was significantly inhibited in a high glucose environment; however, following treatment with SRT1720 and PCSS treatment, levels of secreted IL-8 were significantly increased; furthermore, these levels were markedly higher than in normal EACCs (Fig. 2E). bFGF is a fibroblast growth factor and fibroblasts effectively promote the formation of scars, thereby promoting wound recovery (21). It was demonstrated that the secretion of bFGF by EACCs was significantly inhibited in high glucose; however, following treatment with SRT1720 and PCSS, the secretion of bFGF was restored (Fig. 2F). Notably, the effect of PCSS on bFGF secretion was greater than that of SRT1720. Taken together, these results demonstrate that, although EACCs treated with SRT1720 and PCSS normalize secretion of the four cytokines, PCSS was more effective than SRT1720 at normalizing cytokine secretion. This may be due to the stable release of SRT1720 by PCSS; SRT1720 is released from PCSS at a rate of ~7.14%/day for 2 weeks.

PCSS-treated EACCs promote the proliferation and migration of HUVECs. To verify whether EACCs are able to promote the proliferation and migration of vascular endothelial cells paracrine, EACCs were cultured in high glucose medium (Baseline), or co-cultured in high-glucose medium along with PCS, SRT1720 or PCSS. Subsequently, the conditioned media were collected, and the medium from EACCs cultured in normal medium was collected as a normal control. Collected conditioned media were used to treat HUVECs and determine the effect on the proliferation and migration of HUVECs

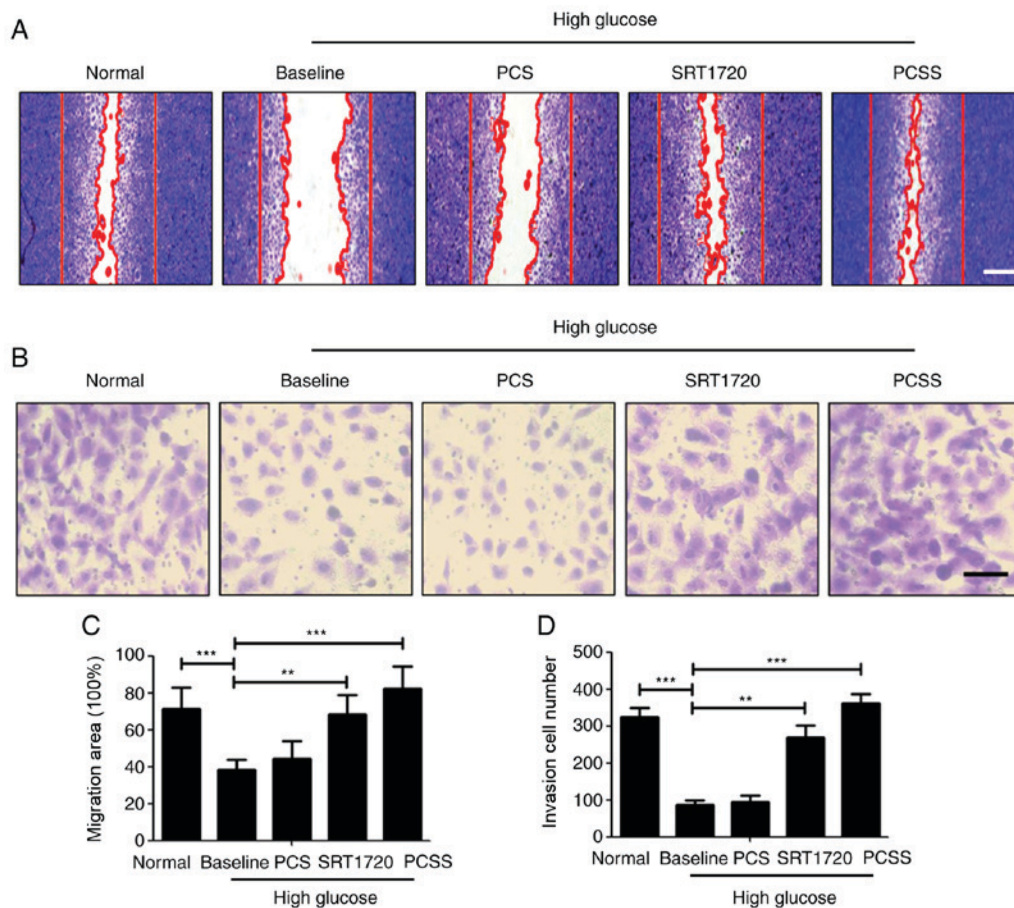

Figure 3. PCSS-treated EACCs promote the proliferation and migration of HUVECs. The conditioned mediums of EACCs treated by different agents were collected to perform scratch wound and invasion assays in HUVECs. The conditional medium of PCSS-treated-EACCs significantly promoted the (A) migration (bar=500 μm) and (B) invasion (bar=100 μm) of HUVECs. Quantification of the (C) scratch wound and (D) invasion assays. **P<0.01 and ***P<0.005. EACCs, embryonic artery cluster of differentiation 133$^+$ cells; PCS, polylactic acid mixed with collagen protein and silk; PCSS, PCS mixed with SRT1720; HUVECs, human umbilical vein endothelial cells.

(Fig. 3). The results of the scratch wound assay indicated that, although HUVECs in the Baseline group underwent migration to a certain degree, the migration rate was much lower than in the normal group. However, treatment of HUVECs with the conditioned media from SRT1720- or PCSS-treated EACCs, the migration of HUVECs increased significantly. HUVECs treated with conditioned medium from PCSS-treated EACCs exhibited the highest rate of migration (Fig. 3A and C). To further examine the invasion of HUVECs following treatments with different conditioned media, a Transwell invasion assay was conducted. The results of this assay were consistent with the results of scratch wound assay; conditioned media from SRT1720- or PCSS-treated EACCs significantly promoted the invasion of HUVECs and the effect of the medium from PCSS-treated EACCs was better (Fig. 3B and D). These results indicated that PCSS significantly promoted the paracrine action of EACCs, thus significantly increasing the migration and invasion of HUVECs. Treatment with conditioned medium from SRT1720-treated EACCs also significantly increased the migration and invasion of HUVECs, but to a lesser extent than PCSS.

PCSS-EACCs promote the healing of diabetic ischemic ulcers. To clarify whether PCSS-treated-EACCs promote the recovery of diabetic ischemic ulcers; a model of diabetic ischemic ulcers was established by performing STZ injections in C57 mice; subsequently, the material containing EACCs was used to cover the wound (Fig. 4A). To investigate the effect of PCSS-treated-EACCs, the C57 mice were split into 6 different groups (all, n=20). At 0, 3, 7 and 14 days following transplant, wound healing was recorded and photographed. The results indicated that the wounds of the normal control mice had completely recovered by day 7. The wound healing process in the mice with diabetes was slower; the wound healing process in mice from the EACC group was markedly inhibited compared with the control group. However, in the PCS-EACCs group, the wound healing process was quicker compared with the EACCs group. Furthermore, SRT1720-EACCs had a more beneficial effect on the wound healing process than PCS-EACCs. The effect of PCSS on wound healing was almost the same as that of PCSS-EACCs. Treatment with PCSS-EACCs had the best effect on the healing time of ischemic ulcers in diabetic mice and the recovery rate of the mice in this group was similar to that of mice in the control group (Fig. 4B and C). These results may be due to the stable release of SRT1720 by PCSS over a prolonged period of time and that SRT1720 is able to promote the biological function of EACCs. These results further

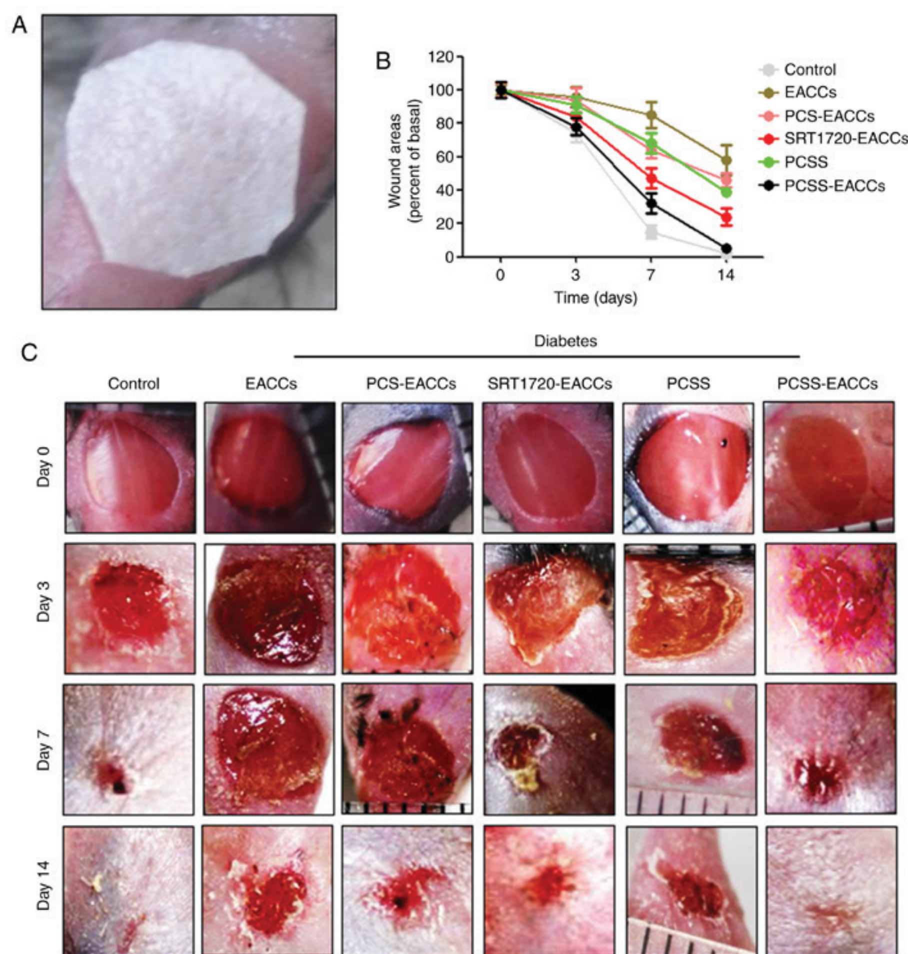

Figure 4. PCSS-EACCs promote the healing of diabetic ischemic ulcers. (A) EACCs were seeded on the PCSS and transplanted into diabetic ischemic ulcers in mice. (B and C) Wound healing in mice was recorded and photographed at 0, 3, 7 and 14 days, and wound areas were quantified. EACCs, embryonic artery cluster of differentiation 133^+ cells; PCS, polylactic acid mixed with collagen protein and silk; PCSS, PCS mixed with SRT1720.

verify the beneficial effect of PCSS-EACCs on the healing of diabetic ischemic ulcers.

PCSS-EACCs promote the angiogenesis of capillaries in the healing of diabetic ischemic ulcers. To further evaluate the effect of PCSS-EACCs on the repair of diabetic ischemic ulcers, ulcer tissues from each group of mice were collected and stained with H&E. The results indicated that the immune response was effectively suppressed following PCSS-EACCs treatment; additionally, the amount of vascularization of capillaries in ulcer tissues was markedly improved following PCSS-EACCs treatment (Fig. 5A). In order to further detect the effect of PCSS-EACCs on angiogenesis in diabetic ischemic ulcers, immunofluorescence staining was performed to detect CD31 expression, which is a marker of vascular endothelial cell (Fig. 5B). The results suggested that SRT1720-EACCs could effectively promote the proliferation of vascular endothelial cells and therefore angiogenesis. The effect of PCSS-EACCs on angiogenesis was better compared with that of SRT1720-EACCs, due to the stable release of SRT1720 by PCSS. Wound capillary density was measured using a microscope (Fig. 5C). The results indicated that angiogenesis was significantly promoted following transplantation with PCSS-EACCs, restoring blood supply and promoting the healing of the diabetic ischemic ulcer.

Discussion

Diabetic ulcers primarily occur in patients with early diabetes that do not exhibit peripheral neuropathy and peripheral vascular disease, but experience foot infections, suppuration or ulcers caused by paronychia or beriberi; the primary symptoms of nerve and vascular diseases (22,23). Diabetic ulcers may be classified as diabetic neuropathic ulcers, diabetic ischemic ulcers and diabetic mixed ulcers; diabetic ischemic ulcers account for ~36% of all diabetic ulcers (24,25). At present dressings seeded with growth factors may be an effective method of treating diabetic neuropathic ulcers; however, there are currently no effective methods of treating diabetic ischemic ulcers (26).

Stem cell therapy may be an effective method of treating diabetic ischemic ulcers (27). Previous studies have demonstrated that EACCs markedly improve the treatment of diabetic ischemic ulcers. However, the survival, growth and biological function of EACCs were inhibited due to the high glucose environment of the ulcers (4-7). Therefore, promoting the survival and function of EACCs in a high glucose environment may improve the therapeutic effect of EACCs against diabetic ischemic ulcers.

The Sirt family serves an important role in regulating the survival and biological functions of cells; the 7 members

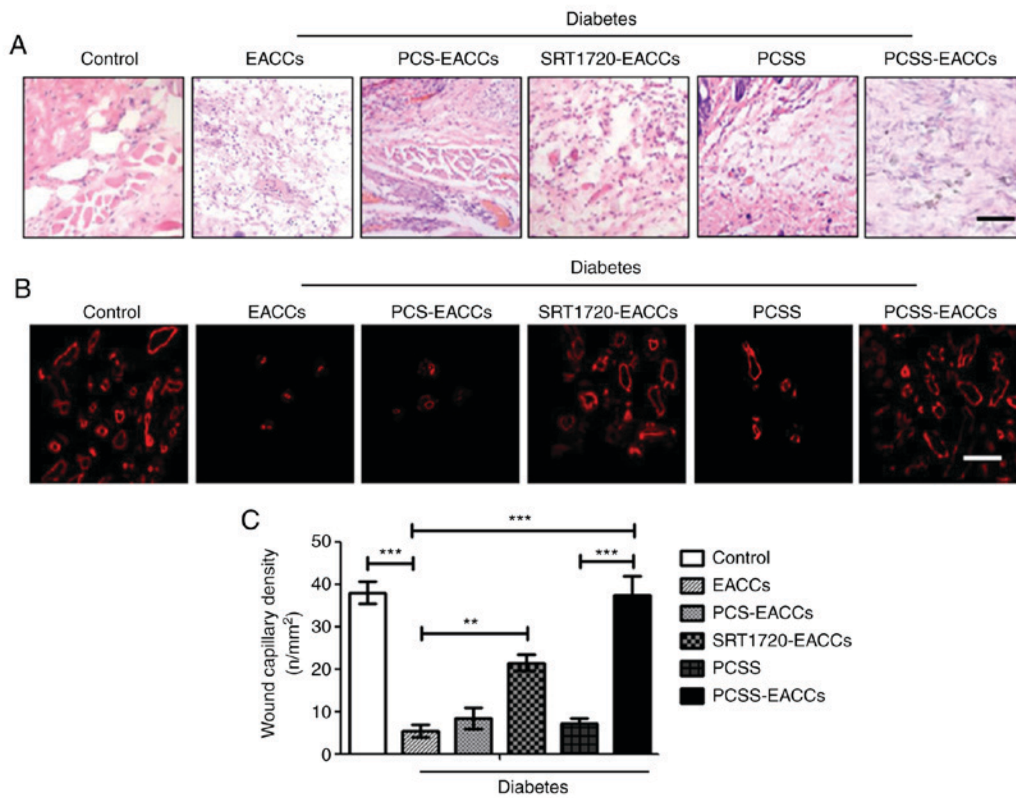

Figure 5. PCSS-EACCs promote the angiogenesis of capillaries during the healing of diabetic ischemic ulcers. Ulcer tissues were collected following 14 days and (A) Hemotoxylin and eosin staining (bar=100 μm) and (B) immunofluorescence staining to detect cluster of differentiation 31 expression (bar=100 μm) were performed and observed using a microscope; subsequently, photographs of vascular density were obtained and the number of capillaries were counted. (C) Quantification of the number of capillaries in ulcer tissues. **P<0.01 and ***P<0.005. EACCs, embryonic artery cluster of differentiation 133$^+$ cells; PCS, polylactic acid mixed with collagen protein and silk; PCSS, PCS mixed with SRT1720.

of the Sirt family regulate cell proliferation, differentiation, senescence, apoptosis and metabolism by interacting with the P53, forkhead box protein 0, Ku70 and PGC-la proteins (28). Sirt1 interacts with PGC-la, which is the cofactor of peroxisome proliferator-activated receptor (PPAR)-γ and, along with NAD, regulates gluconeogenesis-related gene transcription. Furthermore, Sirt1 reduces cellular apoptosis and senescence, and increases the survival rate of cells under oxidative stress through the inhibition of pro-apoptotic factor p53 (29,30). It has been demonstrated that SRT1720 effectively activates the expression of Sirt1 (13). As an agonist of Sirt1, SRT1720 increases metabolism and mitochondrial biogenesis via the transcriptional activation of PGC-1a and PPAR family members, which are peroxisome proliferator-activated receptors. SRT1720 inhibits the immune response and increases insulin sensitivity in type II diabetes; it also inhibits acute oxidant injury and protects mitochondrial function (31). Furthermore, it has been demonstrated that SRT1720 stimulates cell survival and inhibits cellular apoptosis (31,32).

Although treatment with EACCs induces a beneficial effect on diabetic ischemic ulcers, the high glucose environment of the ulcers inhibits the therapeutic effect of EACCs. To determine the effect of Sirt1 on the biological activity of cells, the composite material PCSS was designed using the PLGA collagen protein, silk and SRT1720. PCSS is able to induce the steady release of SRT1720 and also promotes the growth and biological function of EACCs following the seeding of EACCs thus further promoting the proliferation and migration of HUVECs. The results of the animal experiments performed in the current study indicated that transplantation with PCSS-EACCs effectively promotes the healing of diabetic ischemic ulcers. The mechanism of this effect is the release of SRT1720 by PCSS, which activates Sirt1 in EACCs, promotes the growth and paracrine secretion of EACCs and promotes angiogenesis in the local ulcer tissue, thus restoring blood supply and promoting the healing of diabetic ischemic ulcers.

Many people are still suffering from diabetic ulcers and there are currently no effective treatments for diabetic ischemic ulcers (1,2). The current study used Sirt1 as a target molecule. The Sirt1 agonist SRT1720 was selected as a therapeutic drug and the cell growth matrix (PCSS) was designed by combining PLGA, collagen, silk and SRT1720 via electrospinning, which allowed the slow and steady release of SRT1720 over a prolonged period of time. Subsequently, EACCs were seeded onto the matrix (PCSS-EACCs) and transplanted into diabetic ischemic ulcers in mice. The results of these experiments indicated that the angiogenesis in ulcer tissue was markedly improved following the transplantation of PCSS-EACCs, thus improving the healing of diabetic ischemic ulcers. Therefore, the results of the current study suggest that PCSS-EACCs may be developed as a novel and effective method of treating diabetic ischemic ulcers.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

PKC and CLH contributed to the conception, design, writing and revision of the manuscript, PKC and XLC contributed to the acquisition of data, and XXS and XJS contributed to the analysis and interpretation of data.

Ethics approval and consent to participate

All procedures performed in animals were approved by the Animal Care and Use Committee of the Third Military Medical University (Chongqing, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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