Mesh-like Electrospun Membrane Loaded With Atorvastatin Facilitates Cutaneous Wound Healing by Promoting The Paracrine Function Of Mesenchymal Stem Cells

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Abstract

Background

The Functional electrospun membrane was a promising strategy to promote wound healing, however, the microstructure and drug loading of the membranes still need further improvement. Here, we designed a novel mesh-like electrospun fibrous loaded with atorvastatin and investigated the effect on the paracrine secretion of BMSCs and wound healing.

Methods

We fabricated a mesh-like electrospun membrane by using the copper mesh receiver. Physical properties of the membranes were characterized with Scanning Electron Microscopy, Fourier Transform Infrared Spectroscopy, Tensile Strength Analysis and Contrast Angle Test. The drug Release was measured with a concentration curve of certain times. In vitro, we tested the effect of

conditioned-medium (CM) from BMSCs on the migration and pro-angiogenic function of endothelial cells and used the real-time polymerase chain reaction (RT-PCR) to investigate the expression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF) genes in BMSCs. Furthermore, a wound model of rat was established, the atorvastatin loaded mesh-like membranes (PCL/MAT) seeded with BMSCs were used as artificial skin to evaluate the wound-healing efficiency.

Results

The Fourier Transform Infrared Spectroscopy reveals the successful loading of atorvastatin in mesh-like membranes (PCL/MAT). Compared with the random electrospun fibrous (PCL/R) and the mesh-like electrospun fibrous without drug-loading (PCL/M), PCL/MAT has the strongest effect on the migration of HUVECs. The scratches were almost closed after 24h of cell migration in the PCL/MAT group, whereas the scratches maintained a distance for the other groups. The effect of the PCL/MAT group was significant on the pro-angiogenic function of endothelial cells with obviously higher tube length than those of the other groups. And all above corresponding to the increased VEGF and b-FGF gene expression of BMSC in the RT-PCR. In vivo, PCL/MAT seeded with BMSCs significantly accelerated the speed of healing, improved the neovascularization and collagen reconstruction in the wound area compared with the other groups.

Conclusions

Mesh-like topography of fibrous scaffolds with atorvastatin releasing can provide unique microenvironment that benefiting paracrine of BMSCs and migration and pro-angiogenic function of endothelial cells, thus accelerating wound healing. It's a novel strategy to design electrospun membranes for wound treatment.

Keywords

mesh-like topography, electrospun fibrous, atorvastatin, bone marrow stem cells, paracrine

secretion, tissue engineering, wound healing

Figure 1. The experimental designed to investigate the influence of the fiber morphology and atorvastatin (ATV) loaded in the fiber on the paracrine secretion function of BMSCs. The scaffolds for cell culture included electrospun fibers (EFs) in random, a mesh-like organization, and the mesh-like organization with atorvastatin loaded in, which were designated as PCL/R, PCL/M and PCL/MAT, and the cultures on EFs were compared to the BMSCs cultured on polystyrene microplate (MP). Then the PCL/MAT membranes seeded with BMSCs were used as the artificial skin in vivo wound healing.

Background

Skin, one of the most important organs of human body, plays a significant role in sensory detection, absorption, secretion and protecting against external insults, which is very important to the health of the body. [1] The integrity of skin can easily be damaged due to various causes with poor blood supply, it not only threatens the health of the internal tissues under the wound [2] but also be risky for the health of the whole body. [3] The use of autologous skin grafts to cover skin defects with insufficient blood supply is subjected to donor skin shortage. [4] An ideal wound

dressing should be biocompatible, soft, create moist surroundings within the wound area, isolate the wound from outside environment in order to avoid secondary damage and enhance the growth of cells under the wound. [5], [6] Without the help of suitable dressing, the natural process of repair after tissue injury can be unsatisfied with poor skin regeneration. Therefore, clinical skin repair cannot be done well without appropriate dressing.

Tissue engineering is a combination of cells and biocompatible extracellular scaffold materials with appropriate biochemical factors, aims to repair and replace damaged tissues. [7] In recent decades, MSCs are one of the most widely studied cell types in regenerative medicine and have attracted a lot of attention because of its effective and safe role in tissue regeneration. [8] Studies have shown that the paracrine products of MSCs could exhibit a variety of functions including immunomodulation, angiogenesis, anti-apoptosis, anti-scarring, chemoattraction and modulating local stem and progenitor cells. [9], [10] In particular, the proangiogenic factors especially vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF) are secreted by MSCs, can coordinate multifarious biological processes that are required for angiogenesis and tissue regeneration, are of vital importance in wound healing process. [11], [12]

Scaffolds are key components of tissue engineering, serving as a substrate on which cells attach and proliferate,[13] A scaffold with specific structure, surface topography and chemical characteristics could not only create an appropriate and decent environment for cell growth but also affect the secretory function of cells through cell-material interactions. [14]

Atorvastatin (ATV), a HMG-CoA reductase inhibitor used to reduce the blood lipid in clinical, has long half-life period, active metabolites and high protein binding rate. [15] Topical treatment of atorvastatin has been reported to promote tissue regeneration of acute lesions in rats and regulate

cell growth-related proteins and cytokines[16], [17] and has an effect of promoting wound healing, anti-inflammatory, and pro-angiogenesis. [16] It not only has excellent long-term effects but also avoids oral side effects. [17] More importantly, it is reported that exosomes derived from the bone marrow MSC (BMSC) pretreated with atorvastatin (ATV), could exhibit better pro-angiogenic ability in diabetic wound healing and its underlying molecular mechanism. [18] In addition, studies have shown that atorvastatin could protect mesenchymal stem cells from hypoxia and serum deprivation damage and strengthen the paracrine effect of stem cells. [19], [20], [21]

To this end, we here selected electrospun fibers (EFs) to investigate the effect of mesh-like topography combined with ATV on the paracrine function of MSCs. Because of the advantages such as the convenient fabrication process, EFs became an important variety of scaffold materials that haven been extensively studied in tissue engineering[22], [23], [24]

Here, as it is illustrated in Fig 1, polycaprolactone (PCL) EFs with two different arrangement modes (PCL/R and PCL/M) and the mesh-like morphologies loaded with ATV(PCL/MAT) were fabricated as model scaffold materials. The effects of the fibers on rat bone-derived MSCs (BMSCs) to produce pro-angiogenic paracrine factors were investigated. The function of these paracrine factors was further studied through collecting conditioned-medium (CM) from different culture systems and applying it to cultures of endothelial cells in vitro. Finally, in Figure 1, BMSCs were seeded onto the PCL/MAT scaffold to functionalize it as an artificial skin to investigate its effect on promoting wound healing in vivo. Our study suggested that the mesh-like topography combined with atorvastatin could remarkably enhance the paracrine function of BMSCs seeded on it, and PCL/MAT scaffold with BMSCs seeded on demonstrated an excellent reconstruction of skin tissues as tissue engineered skin, with clear potential to promote cutaneous

wound healing.

Methods

Cell culture

The bone marrow stem cells (BMSCs) and the Human umbilical vein endothelial cells (HUVECs, ATCC CRL-1730) were used in this study. BMSCs were isolated from the femurs of the juvenile Sprague–Dawley rats (4 weeks of age). The end of bone was cut off by scissors and the culture medium was added to isolate cells from the bone marrow by density gradient centrifugation. The obtained cells were seeded in a T25 culture flask and cultured in alpha minimum essential medium (α -MEM) containing 10% (v/v) fetal bovine serum (FBS, Gibco, U.S.A.) and 1% (v/v) penicillin-streptomycin at 37°C in a humidified CO₂ incubator. The culture medium was changed every 3 days and use phosphate buffer saline (PBS) to remove unattached cells. Cells were passaged each time when the primary mesenchymal cells reached 80% confluence. Only early passages (p3-p5) of cells were used in this study.

HUVECs were obtained from BeNa Culture Collection and cultured in endothelial cell medium (ECM) with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin at 37°C in a humidified CO₂ incubator. The medium was replaced every 3 days until a confluence of 80% was reached. For the collection of conditioned media, the BMSCs were cultured in FBS-free MEM- α for a prescribed period of time. To detach cells from the culture plates, BMSCs and HUVECs were treated with trypsin/EDTA solutions.

Fabrication of fibrous scaffolds

Electrospun fibrous (EF) scaffolds were prepared from polycaprolactone (PCL) solution. 10% w/v PCL was dissolved in a mixture of CHCl₃ and dimethylformamide at the volume ratio of 9:1.

Then stir for 2 h, until PCL was completely dissolved. To prepare the atorvastatin loaded EF (PCL/MAT), atorvastatin powder (2.5mg, 25mg and 250mg) was added into the PCL solution and stirred for another 1 h. Then 5 mL of the solution was loaded into a syringe with a blunt-ended stainless 21-gauge steel needle. The feeding rate and the applied voltage were controlled at 0.5 mL/h, 12 kV, respectively. The collection distance for random EF (PCL/R) and mesh-like EF (PCL/M and PCL/MAT) was 13 cm. The PCL/R was collected on a flat plate, while the PCL/M and the PCL/MAT on a copper mesh. The EF membranes were cut into predefined sizes and shapes manually. For sterilization, the EF scaffolds were exposed under ultraviolet radiation for 2h on each side in the bio-safety cabinet and were kept in sterile dishes at 4 °C until being used.

Scanning electron microscopy (SEM) images

Topographical features of the scaffolds were examined by scanning electron microscopy (SEM) (S-4800, Hitachi, Japan). The mean diameter was calculated from the scanning electron microscopy (SEM) images with ImageJ.

Fourier transform infrared spectroscopy

To confirm the successful load of ATV in the EFs, the PCL/M, the PCL/MAT and atorvastatin raw powder samples were analyzed via Fourier transform infrared spectroscopy (Nicolet 5700, USA).

The infrared spectra of the samples were measured over a wavelength range of 4000-400 cm⁻¹.

Tensile Strength Analysis

The uniaxial tensile testing of electrospun scaffolds was carried out using a Zwick (model Z020) universal mechanical testing machine with a load cell capacity of 20 kN at a cross-head speed of 5 mm/min for modulus determination. The three groups of fibrous membranes were cut to 40 mm*10 mm pieces with being fixed on the molds, and stretched at a constant speed (5 mm/min)

until the material broke. The tensile strength and Young's modulus were determined from the stress-strain curves.

Hydration behavior

Contact angle test was performed by contact angle goniometer (Krüss, Hamburg, Germany) at room temperature. At least two measurements were conducted at two locations of the scaffold specimen and the contact angle was measured after 10 seconds.

Drug release test

In order to detect the drug release of the samples, multiple mass gradient drugs were added into 50% (v/v) methanol-phosphate-buffered saline (PBS) solutions to prepare a concentration curve for atorvastatin. The atorvastatin-loaded electrospun membranes (PCL/MAT-ATV=2.5mg) were divided into square samples with an average weight of 10 mg. Each membrane sample was immersed into 10 mL of PBS (pH = 7.4) at 37 °C. At predetermined time points, 2 mL of the release medium was added into 2 mL of methanol and then ultrafiltered for detection. The release medium taken out of the original release system was replaced with an equal volume of fresh PBS. The amount of released atorvastatin in the mixture was analyzed by a microplate reader (Thermo, USA) at 246 nm. [25]

Cell Proliferation

Cell proliferation was investigated by the CCK-8 (Dojindo, Japan) assay at different time points.

The BMSCs were seeded on the membrane surfaces (PCL/R, PCL/M, PCL/MAT-2.5mg\25mg\250mg) at a cell density of 1×10^4 cells/well and incubated for 1, 3, or 5 days. Fresh culture medium (300 μ L) and 30 μ L of CCK-8 solution were added to each well, after which the cells were rinsed twice with PBS. After incubation for 2 h at 37 °C, 100 μ L of the supernatant was

transferred to a 96-well plate, and the absorbance at 450 nm was determined using a microplate reader (Thermo, USA). Proliferation of cells was contrasted according to the absorbance of different groups.

Fluorescent Confocal Imaging of Initial Cell Adhesion

For fluorescent confocal imaging, scaffolds seeded with BMSCs (1×10^4 cells/well) were fixed overnight by 2% paraformaldehyde (PFA), and the cells were stained with Rhodamine Phalloidin for 1 h and then DAPI for 5 min. The stained cells were pictured by A1R-si confocal microscope (Nikon, Japan.)

Real time PCR assay

We investigated the expression of VEGF and b-FGF genes in BMSCs using real-time polymerase chain reaction (RT-PCR). After being cultured on the membrane surfaces (PCL/R, PCL/M, PCL/MAT) and MP for 3 days, the BMSCs which were seeded at a density of 1×10^4 cells/mL were prepared for the test. All BMSCs from the samples were washed twice with PBS, and the total RNA was extracted using RNAzol reagent. The RNA concentrations were determined by spectrophotometry. Then, a PrimeScript RT reagent kit was used, in which all RNA was reverse-transcribed into complementary DNA. We carried out RT-PCR on the VEGF gene and b-FGF gene, all gene expression values in one sample were normalized to the GAPDH level. All the primary sequences were listed as follows: GAPDH (Forward, TGAAGGTCGGAGTCAACGGATTTG; Reverse, CATGTGGGCCATGAGGTCCACCAC); VEGF (Forward, TTCATGGATGTCTATCAGCG; Reverse, GCTCATCTCTCCTATGTGCT); b-FGF (Forward, GTCAAACCTACAGCTCCAAGCAGAA; Reverse, AGGTACCGGTTTCGCACACA).

Migration Assay

The chemotactic effects of different CM on HUVECs migration were determined using a wound-healing migration assay in vitro. Briefly, HUVECs cultured with different medium, which include half fresh complete medium and half conditioned medium (PCL/R-CM, PCL/M-CM, PCL/MAT-CM and MP-CM), were seeded in the 6-well culture plates at 1×10^5 cells per well and cultured until reaching about 90% confluence, then the cell monolayer was scratched with a sterile pipet tip to form a wound. HUVECs cultured with normal medium acted as the control. After incubation for 24 h, samples were observed using the bright-field microscopy. The cell motility was quantitated by observing the width of the gap in the healing wound.

Endothelial Tube Formation Assay

For the HUVEC tube formation assay, 1×10^5 HUVECs were seeded onto the Matrigel films in 24-well plates, treated with 50% CM and 50% ECM supplemented with 2% FBS. Cells were incubated for 6 h and imaged under bright-field microscopy. Tube length was quantified by Angio Tool software.

Wound Healing Animal Models

In order to evaluate the wound-healing efficiency, a wound model of rat was established. Twenty-four SD male rats (weighing 180–220 g) were prepared for wound-creating surgery. By general anesthesia with inhaled isoflurane (5%), all dorsal hair of rats was shaved and the wound areas were sterilized with povidone iodide. Then two 10-mm diameter round full-thickness excisional skin wounds were created on each side of the midline using a biopsy puncher following the hair removal from the dorsal skin. It's worth noting that a donut-shaped silicone splint was glued to the skin using the biomedical glue with the wound centered in the splint. The splint was used to stabilize the wound and avoid contraction, making wound area comparable. Each rat was

randomly placed in the four groups. The blank control group received no membrane around the wound, while round (10 mm diameter) piece of membranes (PCL/MAT-BMSCs, Chitosan, Gauze dressing) were wrapped around the repair site and immobilized. The experimental group is the PCL/MAT artificial skin (BMSCs density is 1×10^4 cells per cm^2 seeded on each PCL/MAT member) while the negative control group had gauze dressing around the wound and the positive control group had commercial chitosan dressings around the wound. Animal wound images were collected on a DSLR camera (Canon) 0,7, and 14 days after surgery. The rats were sacrificed on 7 days and 14 days after surgery.

Macroscopic and Histological Evaluation

Histology was conducted by excision of the healthy tissues surrounding each wound following sacrifice of the animal and fixing in 4% paraformaldehyde overnight. Samples were dehydrated in an ascending gradient of ethanol (70%, 85%, 90%, 95% and 100%) then xylene (50% and 100%), embedded in paraffin and sectioned to a thickness of 7 μm (Leica, RM 2245, Germany). All samples were stained with Masson's trichrome (Sigma) or H&E. The stained samples were sealed with resinene and visualized using an inverted microscope (Nikon, TI-S, Japan). Statistical analysis Quantitative data were collected from at least five samples. Data are expressed as means and standard deviation. Groups were compared using ANOVA to evaluate the significance of differences. $p < 0.05$ was considered statistically significant.

Statistics

All data were shown as mean $\pm$ standard error of the mean (SEM). One-way ANOVA was utilized for the evaluation of the significant difference. *P value < 0.05 was considered significant. GraphPad Prism 6 software was applied for the statistical comparison of all the data.

Results

Preparation of electrospun fibrous matrices and co-culture of BMSCs

As shown in Figure 1, three types of EFs, showing random, mesh-like pattern and ATV-loaded mesh-like pattern, which were designated as PCL/R, PCL/M and PCL/MAT respectively. BMSCs were seeded on the membranes and their gene expression of VEGF and b-FGF were analyzed through real-time polymerase chain reaction (RT-PCR) while the function of paracrine products from different culture systems was investigated by collecting CMs and applying them to the in-vitro cultures of HUVECs. Furthermore, the function of PCL/MAT membrane seeded with BMSCs as artificial skin was investigated in vivo skin wound healing model. The function of the paracrine products In vitro experiments, the cells cultured on the polystyrene microplate (MP) were used as a control for comparison to understand how the materials with fibrous topological characteristics may affect the paracrine secretion of BMSCs.

Appearance of the Prepared Membranes

In Fig 2, SEM micrographs showed that the EF scaffolds fabricated through electrospinning processes using different collectors had distinct morphologies Fig 2 (a)–(f). While the PCL/R contained random fibers, the PCL/M and the PCL/MAT collected using the copper mesh as a collector was made of bundled fibers oriented vertically in lattice patterns with the grid length at 650 μm . In addition, the diameters of PCL/R, PCL/M and PCL/MAT fibers were 1.854 ± 0.047 , 1.876 ± 0.023 , 1.782 ± 0.055 μm , respectively. The fiber diameters of all groups reached the micron level. And it is worth noting that the electrospun fibers of each membrane were continuous, round, and nearly bead-free, and the spinning surface of the ATV loaded group is rougher than the unloaded group (indicated by the orange arrow).

Characterization of the Prepared Membranes

In Fig 3 (a), the FTIR spectra are shown for pure ATV raw powder, pure PCL fibers (PCL/M) and ATV loaded-PCL fibers (PCL/MAT). As can be seen in Fig 3 (a), in pure ATV drug spectra, the characteristic peaks were observed at 1450 cm^{-1} (C=C) stretching, 840 cm^{-1} (C-H) stretching, and 1103 cm^{-1} (C-F) stretching, which are related to the phenyl groups in ATV. In the PCL/MAT fiber, those similar peaks appeared, which indicates the successful loading of ATV in PCL/MAT with no similar peak exists in the PCL/M fiber. In Figure 3 (b), the water contact angle of random fibers (PCL/R) was $129.10^{\circ} \pm 0.71$ while the mesh-like scaffolds loaded with and without ATV was $125.30^{\circ} \pm 0.57$ and $132.05^{\circ} \pm 0.92$ respectively. The Young's modulus and tensile stress of EFs were calculated from the stress-strain curves and are presented in Fig 3 (c)-(e) respectively. The PCL/R membrane showed higher Young's modulus and higher tensile stress than PCL/M and PCL/MAT membrane. The results indicated that PCL/M and PCL/MAT were more elastic in the process of

increasing stress and have better flexibility than PCL/R group. ($p < 0.05$).

In Vitro Drug Release

According to an approximate concentration standard curve of atorvastatin, we found that cumulative concentrations of atorvastatin were released from PCL/MAT. As shown in Figure 4, the release rates of atorvastatin from the PCL/MAT membrane in the first day reached around $43.69 \pm 2.13\%$. Then, during the fourth day, more than $65.47 \pm 1.96\%$ atorvastatin had been released from the membrane. In the next 5–7 days, atorvastatin was delivered more slowly and smoothly by the fibers. The platform release rate of the drug-loaded fiber membrane was $89.34 \pm 2.16\%$.

Biocompatibility of Electrospun Fibrous Membranes

To characterize the biocompatibility of the scaffold, BMSCs seeded at a cell density of 1×10^4 cells/well were cultured on different scaffolds with MP used as the control. Results in Fig 5 (a) indicated that the PCL/R, PCL/M and PCL/MAT (ATV=2.5 mg) scaffolds was non-toxic to cells while the PCL/MAT (ATV=25 mg) and the PCL/MAT (ATV=250 mg) have an inhibitory effect on cell growth. It shows that the ATV and PCL mass ratio of 1/200 (2.5 mg: 500 mg) was the best.

Therefore, PCL/MAT samples were finally produced using this most appropriate ratio.

Fluorescence microscopy images in Fig 5(b)-(d) showed that the BMSCs could all adhere and grow on the three EF scaffolds (PCL/R, PCL/M, PCL/MAT). The confocal microscopic studies showed that cells on both PCL/M and PCL/MAT were relatively elongated and form a new cytoskeleton on the membranes by sprouting or extending the pseudopod while the cells on random fibers (PCL/R) appeared more approximately circular than the mesh-like fibers (PCL/M, PCL/MAT).

Migration and Angiogenic Ability of Endothelial Cells

In Figure 6(a), schematic diagram illustrated the effect of PCL/MAT scaffolds on the paracrine action of BMSCs and the experimental methods above. To probe the function of the secretory

products of BMSCs, the serum-free media conditioned by the BMSCs cultured on different scaffolds and microplates were separately collected and applied to the cultures of endothelial cells. We took the culture medium supernatant of different groups (MP, PCL/R, PCL/M, PCL/MAT) from which we had cultured BMSCs for 3 days to complete the migration assay Fig 6 (b) and the angiogenic-related assay Fig 6 (c). As shown in Fig 6 (b), the CMs from EF scaffolds (PCL/R-CM, PCL/M-CM and PCL/MAT-CM) have more obvious effects on promoting the migration of HUVECs compared with that of the microplate group (MP-CM). It is worth noticing that the CM from PCL/MAT group has the most significant promotion effect. The mesh-like groups could be found to improve the migration rate compared with that of random group (PCL/R-CM vs PCL/M-CM). More important, the promotion effect was significantly enlarged after the addition of ATV (PCL/MAT-CM vs PCL/M-CM).

According to the Matrigel experiment Figure 6 (c), the total length of the vessel-like tubes formed by cells of the four groups of CM samples increased compared to the control group, suggesting the trophic effects of the collected CMs. Among the four groups of CM samples, the effect of the CMs collected from EF scaffolds (PCL/R-CM, PCL/M-CM and PCL/MAT-CM) were all significantly more potent compared to the MP-CM. There is also a trend that the mesh-like scaffold with ATV-load (PCL/MAT) facilitated more production of the pro-angiogenic factors from BMSCs with its most significant effect on angiogenesis Figure 6 (f) in comparison to other groups. In order to get rid of the effect of possible ATV released from scaffolds on HUVECs, the action of the leach solution of the ATV-loaded membranes was also used to culture HUVECs in our migration assay and angiogenic-related assay, which was designated as PCL/MAT. Results showed that ATV released from scaffolds could slightly accelerate the migration of HUVECs and increase the total

length of the vessel-like tubes Figure5 (b)-(e).

In Figure 6 (f), the results demonstrated that the expression level of the VEGF gene and b-FGF gene on three types of membrane surfaces are apparently higher than the MP, while the PCL/MAT fiber surface was the highest. In addition, the mesh-like group could also be found to improve the expression levels compared with that of random groups (PCL/R vs PCL/M), and the ATV loaded group was also found to improve the expression level compared with the no drug group (PCL/MAT vs PCL/M). Hence, we found that the sustained release of ATV in the drug-loaded system and the mesh-like characteristics may both increase the gene expression level of VEGF and b-FGF, thereby improving the positive angiogenic effect of the BMSCs seeded on the membrane.

Wound Healing Animal Models

After the in vitro cell study, the wound-healing efficiency of PCL/MAT membranes with BMSCs seeded on was demonstrated by creating full thickness skin wounds on the back of rats in an in vivo experiment. Figure 7 (a) shows the optical images of the wound appearances at 0, 7, and 14 days after treatment with PCL/MAT-BMSCs dressing (PB), Chitosan dressing (CS), Gauze dressing (GZ) or Blank (B). The experimental group is the PCL/MAT-BMSCs artificial skin and the negative control group had gauze dressing around the wound while the positive control group had commercial chitosan dressing around the wound. The blank control group received no membrane around the wound. The wound area was measured at certain times (7 and 14 days) to determine the reduction in the wound area. According to the pixel statistics of the wound image Figure 7 (b), the size of the wound tends to decrease after treatment in all groups. It is worth noticing that the wound healing after PCL/MAT-BMSCs coverage was better than the commercial Chitosan group. The wound area of the PCL/MAT-BMSCs group (PB) at each observed time point (7, and 14 days) was smaller than the other three groups, whereas the blank group and the Gauze group always had a lower wound recovery rate. After a 14-day treatment Figure 7(b), the PCL/MAT-BMSCs group showed a wound-healing ratio of $96.72 \pm 0.48\%$ while the data of Chitosan dressing was $94.18 \pm 0.92\%$, which were higher than those of both Gauze ($84.50 \pm 1.3\%$) and blank groups ($80.00 \pm 4.43\%$).

Figure 7 (a) Images on the day of surgery show that the initial wound covered with different dressings and the posterior images of the wounds at different time points (7 and 14 days) after surgery were sequentially arranged. (b) Statistics of the wound area at different time periods (7 and 14 days), which was used to characterize the wound healing of Blank (B), Gauze dressing (GZ), Chitosan dressing (CS) and PCL/MAT-BMSCs dressing (PB).

Histological analysis

H&E and Masson's trichrome were employed to evaluate the histological signs of the wound-healing process. As shown in Figure 8 (a)-(h), 7 days after the wound was created, the blank group and the gauze group presented poor wound healing, including less angiogenesis and sparsely distributed collagen reconstruction compared with other barrier groups. The vascular images of PCL/MAT-BMSCs groups in the wound area were significantly abundant compared with other groups, arranged in parallel with orientation perpendicular to the surface, which indicates better wound repair of granulation tissue. The red arrow indicates the blood vessel Figure 8 (a)-(d). Moreover, as the Masson images shown in Figure 8 (e)-(h), the wound sites treated by PCL/MAT-BMSCs group showed the densest and the most obvious collagen reconstruction in neatly arrangement than other groups.

Figure 8 (a)-(d) H&E staining images of different groups of wound areas and detailed images of H&E staining corresponding to each group. (e)-(h) Masson's stained images of wound areas in each group and detailed images of Masson's staining corresponding to each group. The red arrow indicates the blood vessel. Blank(B), Gauze dressing (GZ), Chitosan dressing (CS) and PCL/MAT-BMSCs dressing (PB), respectively.

Discussion

MSCs have been studied for decades in tissue-engineering because of their regenerative potential.

[26] Evidences obtained through many years of studies have shown that the transplanted cells would only exist for a relatively short period of time in the host due to the complex host reactions, and the paracrine activity of cells may provide a new effective approach in mediating molecular network within the environment towards tissue regeneration. [27] Studies have proved that the paracrine effects, rather than directly differentiating into new tissues is the major effective therapeutic function of MSCs in cell therapy, where the MSCs answer to the specific regional microenvironment of damaged host tissue. [9], [10] Among those paracrine actions, angiogenesis plays an extremely significant role in tissue engineering and has been attracting growing attention. [28], [29] Angiogenesis is a key step in tissue regeneration which is required to supply plenty of nutrients, oxygen, and circulating progenitor cells to injured tissues. [28] Angiogenesis represents a primary consideration when assessing the trophic effect of MSCs and plays an important role in

tissue repair and regeneration.

The combinatorial use of cells and materials has become a significant approach in cell-based therapies, in which understanding the interaction between cells and materials and the associated mechanisms is essential to develop functional materials for future regenerative medicine. [30] The influence of materials on cells has been reported in several studies, included that the possibility of substantially improving the capacity of MSCs to promote paracrine function by manipulating biomaterial characteristics, such as topography, represents an encouraging approach to improving the efficacy of MSC-based treatments and it is also suggested that the fibrous topography may also be beneficial for increasing the extension of MSCs as well as the relevant cytokine release. [26], [31] Further research had design and fabricate MSCs-seeded-scaffold as tissue engineered skin which had mechanical properties similar to that of natural skin and exhibited high biological activity, strong induction of cell migration and up-regulation of gene expression of relevant factors, resulting in excellent wound healing characteristics. [32]

Yet there is no research about how the combination of mesh-like topographical properties and atorvastatin loaded in might affect the paracrine function of MSCs, although each of them has been confirmed to have an effect on stem cell growth. Our study showed that the paracrine function of BMSCs seeded on the atorvastatin-loaded mesh-like scaffold has greatly increased compared with the random scaffold and the drug-free mesh-like scaffold. In this study, atorvastatin was incorporated into the mesh-like PCL membrane via a simple and efficient mixture electrospinning technology, and this PCL/MAT scaffold creates a synergistic microenvironment for BMSCs seeded on it. It has been observed that the mesh-like topography of fibrous scaffolds can upregulate levels of pro-angiogenic cytokines of BMSCs and the combination of atorvastatin

released from scaffolds has a reinforcing effect as it is illustrated in Figure 9. Furthermore, we use the BMSCs seeded PCL/MAT membrane as tissue engineered skin in vitro wound healing model, which reveals remarkable effect on angiogenesis and collagen reconstruction.

Benefiting from electrospinning technology, these fibers can be conveniently arrayed into mesh-like or random phenotypes to provide specific surface morphologies and mechanical properties.

[33] Previous researchers indicated that specific surface topographies had superior potential in promoting tissue regeneration, owing to the topographic cues to adherent cells provided by the mimicking structures. [26], [33], [34] Therefore, in our work, during the process of electrospinning, the PCL jets were extended under the electrostatic force in the electric field and collected by a copper mesh receiver to constitute mesh-like fiber membranes. [26] Then, the mesh-like and random PCL fiber membranes were applied to the culture of BMSCs. FITC–DAPI stained images in Figure 5 (b)-(d) pronounced that relative to the random mat, the BMSCs cultured on mesh-like membranes were relatively elongated and formed a new cytoskeleton by sprouting or extending the pseudopod spread with morphology diversification. The above results confirmed that BMSCs could regulate their growth direction channeled by mesh-like topographies, which provided an important basis for guiding the BMSCs growing. Through profiling the gene expression of important cytokines that are relevant to the pro-angiogenic function of MSCs and in vitro functional assay of the conditioned-media, our study indicates that the mesh-like topography provided unique microenvironment that could enhance the paracrine secretion of BMSCs. The effect of the CM on the tube formation and endothelial migration may be related to the increased gene expression of b-FGF and VEGF factors which were observed in the real-time polymerase chain reaction test.

Besides the specific structure elements of scaffolds, the stimulation of chemical signals from the membrane was also crucial to the paracrine secretion of BMSCs. In this study, atorvastatin, which had been certified to promote wound healing [16], was chosen to enhance the bioactivity of the mesh-like PCL membrane. Moreover, the multiple side effects of oral statins, including myopathy and liver toxicity had also been proposed by some pharmacological studies [35], topical drug delivery system is suggested to be a reasonable alternative way to oral statin in wound healing, as it provides better drug delivery with prolonged action and less side effects. [36] Via a simple and efficient mixture electrospinning technology, atorvastatin was incorporated into the mesh-like PCL membrane, FTIR analyses showed successfully loading of atorvastatin Figure 3 (a). As the release of atorvastatin drugs from the fibers could be regulated by the pores on the fibers, the sustained-release system which could deliver atorvastatin for at least one week. Atorvastatin has an interesting effect on BMSCs growth, as it is reported in some studies that atorvastatin prevents mesenchymal stem cells from harsh microenvironment, for example hypoxia and serum-free injury, through activating amp-activated protein kinase. [19] Moreover, some studies also reported that atorvastatin not only improves the efficacy of MSC transplantation after acute myocardial infarction (AMI) but also improves MSC implantation on therapeutic effects in the acute kidney injury. [37], [38], [39] In terms of the effect of atorvastatin on the paracrine secretion of BMSCs, the mRNA expression and the secretion of VEGF was enhanced in MSCs cultured on statin-loaded scaffolds. [21] What's more, exosomes derived from atorvastatin-pretreated MSCs could accelerate diabetic wound repair by enhancing angiogenesis via AKT/eNOS pathway. [18] And in our study, BMSCs seeded on the drug loaded group reveal increased gene expression of b-FGF and VEGF factors Figure 6 (f). Moreover, the effect of the CM from BMSCs on the tube

formation and endothelial migration may also indicate the influence of atorvastatin Figure 6 (b)-(e). It is interesting to notice that in order to rule out the effects of possible ATV released from scaffolds in the CMs on the migration and pro-angiogenic function of HUVECs, the action of the leach solution of the ATV-loaded membranes was used to culture HUVECs, the results in our study showed that the ATV released from scaffolds could not only increase the total length of the vessel-like tubes but also accelerate migration of HUVECs. Recent study showed that atorvastatin could increase the length of the tube-like vasculature and recover angiogenesis function of Ang II pre-treated HUVECs [40], while some other studies indicated that atorvastatin treatment could increase VEGFA protein levels, proliferation, angiogenesis as well as migration of endothelial cells. [41], [42] In vivo, topical atorvastatin therapy is useful for healing the wounds with remarkable improvement in the skin histological architecture, besides anti-inflammatory effect, statins can modulate cellular processes, such as apoptosis, proliferation, and migration, which makes them promising therapy for wound healing. [17], [43], [44]

Beyond promoting paracrine secretion, isolating harmful threats from the outside world and constructing a stable vascular repair environment for the wound site are of vital importance for promoting local angiogenesis. As an ideal wound tissue engineering barrier, it should be biocompatible and could provide an appropriate environment for cell adhesion and proliferation. [45], [46] What's more, a biological wound tissue engineering barrier is required to have reliable mechanical properties and be better with chemical characteristics which were effective in wound healing. [14], [47] In our work, PCL was fabricated as mesh-like membrane loaded with atorvastatin, BMSCs were seeded on the membrane to be a wound barrier. Like other electrospun scaffolds [48], [49] the PCL membranes in this study possessed a high specific surface area, high

aspect ratio, and high microporosity. [45] Thus, it could not only provide similar architecture to the natural ECM and skin environment, but also deliver cytokines and atorvastatin to the wound area. [45] Besides, excellent tensile mechanical strength [50], ensured reliability of this PCL membrane Figure 3 (d).

Moreover, the reasonable fiber microstructure and excellent biocompatibility leading to improved cell adhesion, migration, and proliferation are also crucial elements for a wound barrier. Research of electrospinning over the past decade had brought solid evidence that PCL was a biosafety material. [51], [52], [53] As illustrated by in vitro tests in Figure 5 (a), no significant cytotoxicity was found in PCL/R、PCL/ M and PCL/MAT(ATV=2.5mg) groups, and DAPI–FITC stained images in Figure 5 (b)-(d) showed that BMSCs cultured on membranes have a normal cell morphology with some cellular antennae and pseudopods which means that cells could adhere and proliferate on the surface of each group successfully. It is worth noting that the normal adhesion and growth of BMSCs were the important pre-condition for paracrine secretion and wound healing. [32], [31] In addition, we established a wound-healing model to estimate the influence of PCL/MAT-BMSCs membrane on wound areas. The wound area of the PCL/MAT-BMSCs group at each observed time point (7, and 14 days) was smaller than the commercial chitosan dressing group, gauze dressing group and blank group Fig 7 (a)(b). In Figure 8 (a)-(h), histological analysis using H&E and Masson's trichrome staining revealed that the animals covered with the PCL/MAT-BMSCs dressing had the highest degree of angiogenesis and the densest collagen reconstruction compared with the other three groups. These may lead to better wound recovery. In summary, the reasonable microstructure, excellent biosafety and efficient biochemical factors made the PCL/MAT-BMSCs dressing reliable as a barrier protector for vascular regeneration and

collagen reconstruction in the wound site.

Conclusion

The mesh-like topography combined with atorvastatin loaded in can work together to provide a unique microenvironment to upregulate levels of pro-angiogenic cytokines of BMSCs that exhibited functions in vitro assays and therapeutic effects in a full-thickness wound healing model. More important, the BMSCs seeded PCL/MAT membrane functioned as an artificial skin to demonstrate repair functions in vivo, which had a therapeutic effect on wound healing and skin tissue regeneration. The design clarified novel design aspects of cell-material interactions in which the topographical characteristics and drug chemical characteristics of materials can be further combined to establish cell signaling or communication networks that take advantage of the paracrine actions of BMSCs to promote specific tissue regeneration, which elucidating a new aspect of fundamental cell-material interactions that has important implications for improving the performance of cell-based therapeutic constructs in vivo.

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Abbreviations

BMSCs: bone marrow stem cells

HUVECs: Human umbilical vein endothelial cells

ATV: atorvastatin

CM: conditioned-medium

EFs: electrospun fibers

MP: microplate

PCL/R: Polycaprolactone random electrospun fibrous

PCL/M: Polycaprolactone mesh-like electruspun fibrous

PCL/MAT: Polycaprolactone mesh-like membranes loaded with atorvastatin

RT-PCR: real-time polymerase chain reaction

VEGF: vascular endothelial growth factor

b-FGF: basic fibroblast growth factor

Authors' contributions

JY.X. and L.Z. designed the studies. JY.X., YL.X. and YF.Z. carried out the research, LF.X., Y.C. and W.Z. assisted in performing research. L.Z., DY.C. and M.W. analyzed the data and wrote the manuscript. L.G. and L.C. reviewed and revised the manuscript. The authors approved the final version of the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All animal procedures were approved by the Experimental Animal Welfare Ethics Committee, Zhongnan Hospital of WuHan University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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