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Astragulus polysaccharide-loaded fibrous mats promote the restoration of microcirculation in/around skin wounds to accelerate wound healing in a diabetic rat model

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ABSTRACT

Tissue engineering scaffolds (TES) can carry numerous biomacromolecules and cells, and they have been widely used in diabetic skin wound healing with positive effects. However, the bioactive retention of biomacromolecules and cells during fabrication and storage is still a factor restricting their use. Moreover, impaired blood supply in/around poorly healing diabetic skin wounds has not been considered. In the present study, a bioactive natural substance of Astragalus polysaccharide (APS), which has stable and confirmed effects on endothelial protection, was embedded into fibrous TES by electrospinning. The administration of APS-loaded TES on the skin wound in a diabetic rat model led to a dose-dependent promotion in skin blood flow around wounds and an increase in endoglin expression and microvessel density in regenerated skin tissues. Furthermore, the higher loading of APS in TES led to faster collagen synthesis, appendage and epidermal differentiation, and wound closure. In summary, the combination of APS with TES is a potentially novel therapeutic strategy for diabetic skin wound healing, as it not only mimics the ultrastructure of extracellular matrixes but also restores skin microcirculation.
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1. Introduction

As a frequent endocrine and metabolic disease, diabetes has become a grave public health problem. The number of global diabetics has surged to 400 million [1]. Diabetic ulcers have become one of the major factors causing disability and death, and they occur in 15% of diabetic cases [2]. Although vasculopathy, neuropathy, and wounding in diabetes are recognized to be the major causes of incurable wounds, glycolipid dysbolism resulting in vasculopathy and neuropathy existed prior to the wound [3,4]. Above this, vascular dysfunction is also a major contributor of neuropathy in diabetes [5]. Although the mechanism of microvascular dysfunction in diabetic ulcers is uncertain, reduced capillary blood flow is a common phenomenon. According to the capillary steal syndrome hypothesis, the increase in total peripheral blood flow and the loss of vasoconstriction in peripheral sympathetic denervation in diabetes results in the shunting of blood away from the capillaries through arteriovenous shunts [3]. These functional and structural microvascular changes result in a reduction of nutrition to the skin.

Astragali Radix (AR) is an important tonifying herb in China that is derived from the dried root of *Astragalus membranaceus* (Fisch.) Bge. (family Leguminosae). In China, it has been used for ulcer healing for hundreds of years. The water-soluble extract of AR has been shown to play a preeminent role in anti-inflammation and fibroblast proliferation, either in cell lines or in primary cultures from diabetic ulcer patients [6–8]. An AR contained Chinese 2-herb formula had been reported to boost the levels of circulating endothelial progenitor cells for local wound vessel incorporation; to augment blood vessel density, vascular endothelial growth factor and endothelial nitric oxide synthase (eNOS) expression; and to attenuate tissue oxidative stress in the wound healing of diabetic rats [9,10]. Astragalus polysaccharide (APS) mainly consists of D-glucose, D-galactose, L-arabinose, and dextrans [11]; it is the most bioactive ingredient in AR and is largely responsible for the pharmacologic actions of anti-inflammation, anti-oxidation,

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immunological regulation, and protection from acute endothelial dysfunction of AR [12–15]. Thus, APS should be appropriate for diabetic wound healing.

The traditional treatment for diabetic skin wounds is strict blood glucose control combined with anti-infection strategies. However, these treatments have little effect on severe ulcers with full-thickness loss and widespread damage, due to the scarcity of the cell growth microenvironment. Tissue engineering scaffolds (TES) have extracellular matrix (ECM)-like architecture, a large surface-to-volume ratio, high porosity and interconnected pores and are considered to be an effective strategy to mimic an environment conducive to tissue regeneration. Although several techniques exist to fabricate a three-dimensional framework with high porosity and pore interconnection, electrospinning is currently the only technique that allows the fabrication of continuous fibres with diameters ranging from several micrometres down to a few nanometres, which are architecturally similar to the structure of the ECM [16]. The extremely high surface-to-volume ratio allows cell attachment, the microscale interconnected pores allow the transport of oxygen and nutrients, the loose bonding between fibres allows cell migration, and the storage and controlled release of active molecules to guide cellular behaviours and tissue assembly are all outstanding advantages of electrospun TES [17]. In our previous studies, the basic fibroblast growth factor and its expressed gene were loaded in the electrospun TES and exhibited excellent promotion of diabetic skin wound healing [18,19]. However, the preservation of cytokine and gene of during the fabrication of TES is still a restricting factor. Natural medicine is another class of bioactive substances that could be embedded in electrospun fibres. Ginsenoside rg3 [20–22] and Shikonin [23] have been easily encapsulated in polymer fibres by electrospinning and have been applied to skin wound healing.

In the current study, TES with varying loading amounts of APS were fabricated by electrospinning and were then administered to a skin wound in a diabetic rat model. The effects of APS-load TES on the restoration of microcirculation were evaluated by the detection of skin blood flow (SBF) around the wound and by immunohistochemically (IHC) staining for endoglin (CD105) expression and counting microvessel density (MVD) in regenerated tissues. The wound healing processes were evaluated by haematoxylin-eosin (HE) and Sirius red (SR) staining for fibroblast proliferation and collagen synthesis and by macroscopic observation of wound closure.

2. Materials and methods

2.1. Materials

Poly(lactide-co-glycolide) (PLGA, lactide:glycolide = 65:35, M_w = 62 kDa) was purchased from Puluo Jiayuan Biomedical Material Limited Company (Zhejiang, China). APS (purity > 85%) was extracted from AR (Fig. 1a) in our laboratory by a water extract-alcohol extraction method [24], purified by sevag deproteinisation [25], and quantified by a phenol–sulphuric acid method [26]. The streptozotocin (STZ) and SR were from Sigma–Aldrich (St. Louis, MO). Rabbit anti-mouse antibody for CD105, goat anti-rabbit IgG-horseradish peroxidase (HRP) and 3,3′-diaminobenzidine (DAB) were purchased from Biosynthesis Biotechnology Co., Ltd (Beijing, China). All other chemicals and solvents were analytical grade and obtained from Zhongshi Chemical Engineering Company (Shanghai, China).

2.2. Preparation of APS-loaded TES

The APS was dissolved in distilled water with carboxymethylcellulose sodium (CMC-Na), and the solution was dropped into PLGA/dichloromethane solution (7%, w/v) followed by vigorous stirring. A water-in-oil emulsion was shaped and added to a 2 ml glass syringe. Emulsions E1, E2, and E3 were prepared with the following ratios of APS to PLGA: 5.5%, 1.5%, and 0.15% (w/w), respectively. The resulting APS-loaded fibres were prepared as described elsewhere [19]. Briefly, the emulsions were electrospun by a typical electrospinning setup assembled in our laboratory, contained within a high-voltage statitron (DC 30,000 V, Tianjin Dongwen High Voltage Power Supply Co., Ltd., Tianjin, China) and a precision pump (LSP01-1A, Baoding Longer Precision Pump Co., Ltd., Hebei, China). The electrospinning parameters of voltage, exit orifice diameter, nozzle velocity, and receiver distance were 15 kV, 0.6 mm, 1.0 ml/h, and 10 cm, respectively. The resulting APS-loaded fibres were termed APS_H-F, APS_M-F, and APS_L-F. The control fibre sample without APS entrapment but with CMC-Na was also prepared as described above and termed Blank-F. The obtained TES were vacuum-dried at room temperature for 2 days to remove any solvent residue.

2.3. Characterization of TES

The TES of APS_H-F, APS_M-F, APS_L-F, and Blank-F were mounted on metal stubs, sputter-coated with gold and then observed by scanning electron microscopy (SEM, FEI Quanta 200, The Netherlands). The diameter of each fibre sample was calculated by using Adobe Photoshop CS, using five SEM images.

The loading amount of APS in TES was determined by extracting the polysaccharide content from the TES. Briefly, a known amount of TES (ca. 100 mg) were dissolved in chloroform (500 μl) and extracted three times with double-distilled water (600 μl). The APS content of the extracted solution was determined by ultraviolet-visible spectrophotometry (UV-2550, Shimadzu, Japan) based on the phenol–sulphuric acid method [26], in which the concentration was obtained using a standard curve from known concentrations of APS solutions. The extraction efficiency was calibrated by adding a certain amount of APS and CMC-Na solution into a PLGA/chloroform solution along with the same concentration outlined in Section 2.2 and extracted using the aforementioned process.

The pore size between fibres was measured using ImageJ software. For each TES sample, at least five SEM images were randomly captured [27]. As the bulk density of the PLGA is known as 1.25 g cm^3, the apparent density and porosity of TES could be calculated using the following equations [28] in which the thickness was measured in micrometres:

$$\text{Apparentdensity}(g \cdot cm^{-3}) = \frac{\text{TESmass}(g)}{\text{TES thickness}(cm) \times \text{TES area}(cm^2)}$$

$$\text{Porosity} = \left(1 - \frac{\text{apparentdensity}(g \cdot cm^{-3})}{\text{BulkdensityofPLGA}}\right) \times 100\%$$

2.4. Creation of skin wounds and treatment with TES

All animal procedures were performed in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Committee of Anhui University of Chinese Medicine. The type I diabetic rat model was established as described in previous research [29]. Briefly, male Sprague-Dawley rats from Medical University of Anhui weighing 250–300 g at 10 weeks of age were given a single intraperitoneal injection of 45 mg/kg STZ, which was dissolved in sodium citrate buffer (pH 4.5). The blood glucose level was monitored every three days after modelling using a complete blood glucose monitor (Shenzhen Radiant Inno-vation Co., Ltd., Shenzhen, China). The STZ-treated rats with glucose levels higher than 16.7 mM were con-

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