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Electrospun tilapia collagen nanofibers accelerating wound healing via inducing keratinocytes proliferation and differentiation

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ABSTRACT

The development of biomaterials with the ability to induce skin wound healing is a great challenge in biomedicine. In this study, tilapia skin collagen sponge and electrospun nanofibers were developed for wound dressing. The collagen sponge was composed of at least two α-peptides. It did not change the number of spleen-derived lymphocytes in BALB/c mice, the ratio of $CD4^+/CD8^+$ lymphocytes, and the level of IgG or IgM in Sprague-Dawley rats. The tensile strength and contact angle of collagen nanofibers were 6.72 ± 0.44 MPa and $26.71 \pm 4.88°$, respectively. They also had good thermal stability and swelling property. Furthermore, the nanofibers could significantly promote the proliferation of human keratinocytes (HaCaTs) and stimulate epidermal differentiation through the up-regulated gene expression of involucrin, filaggrin, and type I transglutaminase in HaCaTs. The collagen nanofibers could also facilitate rat skin regeneration. In the present study, electrospun biomimetic tilapia skin collagen nanofibers were successfully prepared, were proved to have good bioactivity and could accelerate rat wound healing rapidly and effectively. These biological effects might be attributed to the biomimic extracellular matrix structure and the multiple amino acids of the collagen nanofibers. Therefore, the cost-efficient tilapia collagen nanofibers could be used as novel wound dressing, meanwhile effectively avoiding the risk of transmitting animal disease in the future clinical apllication.

1. Introduction

Wound healing is a multifactorial process including inflammatory reaction, re-epithelialization, collagen deposition and angiogenesis. In order to protect the wound area and accelerate injured skin regeneration, safe wound dressings are expected to have excellent biological properties and the ability to induce tissue regeneration. It has been demonstrated in numerous studies that mammal collagen (especially porcine or bovine collagen) has excellent biocompatibility [1–3]. Therefore, this collagen has been extensively used for skin wound healing. However, mammal collagen still presents a risk of transmitting animal diseases, such as bovine spongiform encephalopathy and foot-and-mouth disease [4,5]. Additionally, the application of mammal collagen is restricted because of religious reason [6,7]. Recently, marine collagen has gradually attracted attention because of its abundance and low price. In 2008, Sankar et al. prepared collagen sheet from discarded fish scales (*Lates calcarifer*) that had enough tensile strength (2 MPa) for use as a wound dressing material [8]. Besides, fish collagen compounded with elastin or plant extracts had good biocompatibility with skin cells [4,9,10]. However, the immunogenicity of fish collagen and its biological functions for skin regeneration are still unknown. Thus, of great significance is to study these issues which are directly associated with future clinical applications of fish collagen as a wound-dressing material.

In recent years, studies focused on marine collagen have indicated that the denaturation temperature of tilapia fish scale collagen hydrogels reached 48 °C and increased to 62 °C after crosslinking [11], which was higher than body temperature and suggested good thermal stability. In 2012, Terada et al. constructed a chitosan-fish scale collagen composite scaffold and found that this scaffold was suitable for the attachment and proliferation of mucosal keratinocytes in an ex vivo-produced oral mucosa

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equivalent [12]. Wang et al. also reported that deep-sea redfish skin had the highest contents of protein compared to scale and bone [13], which suggested that fish skin maybe optimal for the extraction of collagen and would have a high application value. In 2014, preliminary studies related to the biocompatibility of tilapia skin collagen were conducted by Yamamoto et al. [14]. However, it is unclear whether tilapia skin collagen could be prepared as a wound dressing with excellent biological effects to induce skin regeneration effectively.

It was known that human keratinocytes (HaCaTs) are one of the key cells involved in skin wound healing. The proliferation and differentiation of keratinocytes play important roles in wound re-epithelialization. Therefore, it is of great importance to study the biological effects of wound dressing on human keratinocytes and validate its healing effects in vivo, which are crucial to assess the potential of new materials for clinical application. Besides, biomimetic environment was also requried for tissue regeneration. Nanofibers fabricated by eletrospinning have adjustable diameters and mimic the structure and function of native extracellular matrix (ECM), which are beneficial for cell adhesion and proliferation. If tilapia skin collagen could be prepared as nanofibers by electrospinning, it might be helpful for its future application.

In the present study, high-purity fish collagen sponge was extracted from tilapia skin through a series of processing and purification technologies. Its amino acid composition and thermal denaturation temperature were analyzed, and the immunogenicity of collagen sponge was evaluated from the perspectives of humoral and cellular immunity. In addition, collagen nanofibers were further prepared by electrospinning. The morphological structure, tensile strength, thermal stability, swelling property and hydrophilicity of the collagen nanofibers were characterized. Furthermore, HaCaTs were chosen to investigate the effects of collagen nanofibers on promoting cell adhesion and proliferation. The mechanisms involved in collagen nanofibers inducing epithelium regeneration at the gene levels were investigated, focusing on the associations between HaCaTs differentiation and the expression of involucrin, filaggrin and type I transglutaminase. Finally, Sprague-Dawley (SD) rat models with full-thickness skin defects were used to confirm the ability of collagen nanofibers to accelerate wound healing. This research attempted to develop a novel biomimetic wound dressing with the function of inducing tissue regeneration, which also provides scientific basis for the future development and application of fish collagen in biomedicine.

2. Experimental

2.1. Preparation of tilapia collagen sponge

Tilapia skin (provided by Shanghai Fisheries Research Institute) was washed, chopped, and stirred in 0.1 M NaOH solution for 1–2 days. The samples were then soaked in 0.5–1 M acetic acid for 4–8 h with continuous stirring. The supernatant was collected by centrifugation and followed by the addition of 0.1–0.5% pepsin with stirring for 24–48 h. Next, 0.4 M ammonium sulfate was added and the precipitate was collected by centrifugation at 10,000*g*. The precipitate was then dissolved in 0.5–1 M acetic acid, dialyzed, and lyophilized to obtain collagen sponges for subsequent use. All procedures were carried out at 0–4 °C.

2.2. Characterization of tilapia collagen sponge

The purity and molecular weight of the tilapia collagen sponge were determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (GE Healthcare, SE 250, USA). The amino acid content of the collagen sponge was determined using Hitachi Amino Acid Analyzer (Hitachi, Japan). The denaturation temperature of the collagen sponge dissolved in 0.1 M acetic acid solution was measured with differential scanning calorimetry (DSC) (204 F1, Netzsch, Germany).

2.3. Lymphocyte proliferation assay

The experimental protocol was approved by the Animal Care and Experiment Committee of the Ninth People's Hospital affiliated to the School of Medicine, Shanghai Jiao Tong University. BALB/c mice were anesthetized using sodium pentobarbital and the spleens were collected under sterile conditions. The lymphocyte suspension was collected by adding red blood cell lysis buffer and diluting to 1×10^6 cells/mL. Lymphocytes were seeded on tilapia collagen sponges in 24-well plates. They were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. In addition, lymphocytes seeded on cover slips without collagen sponge were used as negative control. Lymphocytes seeded on cover slips with the addition of concanavalin A (ConA) were used as positive control. After culture for 7 d, Cell Counting Kit-8 (CCK-8) solution was added, and the lymphocytes were further incubated for 3 h. The optical density (OD) values at 570 nm were measured using a spectrophotometer.

2.4. Subcutaneous implantation assay in SD rats

A total of eight healthy male 6–8-week-old SD rats (200–250 g) were used in this study. The experimental protocol was approved by the Animal Care and Experiment Committee of the Ninth People's Hospital affiliated to the School of Medicine, Shanghai Jiao Tong University. Eight rats were randomly divided into two groups. After anesthetization by sodium pentobarbital, the dorsal skin was longitudinally transected under sterile conditions. The subcutaneous tissues were separated, a tilapia collagen sponge with an area of approximately $1 \times 1\ cm^2$ was implanted, and the skin was then sutured. The control group received a sham operation without the implantation. Twenty-eight days after the operation, the SD rats were anesthetized, and blood samples were collected by cardiac puncture. The concentrations of IgG and IgM in the serum were detected using enzyme-linked immunosorbent assay (ELISA) reagent kits (Tbdscience, China), and $CD4^+/CD8^+$ T lymphocytes were counted by flow cytometry (FCM) (Guava easycyte, Millpore, Germany).

2.5. Fabrication and characteration of electrospinning tilapia collagen nanofibers

The tilapia collagen sponge was dissolved in a hexafluoroisopropanol (HFP) solution (Fluorochem Ltd, UK) with the weight concentration of 8%. The polymer was then placed in a plastic syringe and inserted in a syringe pump (789100C, Cole-Parmer, USA). The voltage and flow rate during electrospinning were 16–18 kV and 1.0 mL/h, respectively. The distance from the needle to the aluminum foil collector was 10–15 cm. The electrospun tilapia collagen nanofibers formed membranes and were further crosslinked using glutaraldehyde vapor and stored in a vacuum-drying oven. The morphology of the collagen nanofibers was observed using scanning electron microscopy (SEM) (JEOL JSM-5600, Japan). The mean fiber diameter and pore size were determined with image analysis software (Image-J, National Institutes of Health) and calculated by selecting 100 fibers or pore areas randomly. The chemical structure of tilapia collagen nanofibers was determined by Fourier transform infrared spectroscopy (FTIR) (Avatar 380, USA). The tensile strength was analyzed using a universal materials testing machine (H5K-S, Hounsfield, UK). The weight loss temperature was determined with a