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Bone marrow-derived mesenchymal stem cells laden novel thermo-sensitive hydrogel for the management of severe skin wound healing

Zhang Lei[a,1], Gurankit Singh[b,1], Zhang Min[a], Chen Shixuan[a], Kaige Xu[b], Xu Pengcheng[a], Wang Xueer[a], Chen Yinghua[a], Zhang Lu[a,c,*], Zhang Lin[a,**]

[a] Guangdong Provincial Key Laboratory of Construction and Detection in Tissue Engineering, Guangdong Provincial Key Laboratory of Proteomics and Key Laboratory of Transcriptomics and Proteomics of Ministry of Education of China, School of Basic Medical Sciences, Southern Medical University, Guangzhou, 510515, China
[b] Department of Mechanical Engineering, Biochemistry and Medical Genetics, University of Manitoba, Manitoba Institute of Child Health, Winnipeg, MB R3T 2N2, Canada
[c] Elderly Health Services Research Center, Southern Medical University, Guangzhou 510515, China

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ABSTRACT

Bone marrow-derived mesenchymal stem cells (BMSCs) are easy to collect and culture, and it is identified that it has multi-directional differentiation potential, moreover it has low immunogenicity, hence it can be used as an allogeneic cell source for skin wound healing. Hydrogel has been widely used in skin wound healing own to it is able to mimic the 3D microenvironment of cells, which supports cell proliferation, migration and secretion. In this study, we created a novel biocompatible thermo-sensitive hydrogel to carry BMSCs for full-thickness skin wound healing. The thermo-sensitive hydrogel loaded with BMSCs can fast achieve sol-gel transition after implanting to the wound. Histological results confirmed that hydrogel-BMSCs combination group showed significant promotion of wound closure, epithelial cells' proliferation and re-epithelialization, and reduced inflammatory responses in the wounds and in the tissues surrounding the wounds. The combination therapy also can promote collagen deposition, TGF-β1 and bFGF secretion and tissue remodeling. The present study provides a promising strategy for the clinical treatment of skin wounds.

1. Introduction

Skin is the largest organ of the human body and is the first barrier to the external environment. Skin wound healing is a common problem and a major challenge for large-area trauma, burn and no healing diabetic skin wound [1]. In recent years, the effects of stem cell transplantation on skin wound healing have been extensively studied [2,3]. Researchers have proven that bone marrow stromal cells (BMSCs) have a positive effect on promoting wound healing. The current researches showed that BMSCs used as seed cells for transplantation, it can secrete various types of cytokines and growth factors, such as transforming growth factor beta 1 (TGF-β1) and basic fibroblast growth factor (bFGF), which can promote the formation of granulation tissue in wounds, assist in the regeneration of skin appendages after epithelization [4–6]. BMSCs can also differentiate into vascular endothelial cells, epidermal cells and other skin appendage structures to participate in wound repair [7–11].

Absorbent cotton medical gauze is primarily used as a conventional dressing for clinical applications. Despite its wide use, gauze has a number of disadvantages. For example, its absorbency is not sufficiently high, it needs to be frequently changed, it is prone to adhere to wounds, and it can lead to secondary trauma. Biological dressings mainly include freeze-dried pork skin and amniotic membranes, although they have significant advantages over conventional absorbent cotton medical gauze, biological dressings still have insurmountable disadvantages, including their limited application, the risk of spreading bacterial, fungal and viral diseases, and the possibility of immunological rejection [12–15].

As an alternative, thermo-sensitive hydrogels can prevent the loss of water and body fluid from wounds, additionally, thermo-sensitive hydrogels can not only adhere to irregularly surfaced wounds but can also resist the invasion of bacteria. Due to their good water-absorption capacity and degradability, thermo-sensitive hydrogels can avoid secondary trauma during the dressing change [16–19]. Furthermore, thermo-sensitive hydrogels provide a temporary support substratum for cell transplantation, as the porous internal structure of thermo-sensitive hydrogels provides a suitable space for cell proliferation and differentiation and for nutrient exchange [20–23].

* Correspondence to: Z. Lu, Elderly Health Services Research Center, Southern Medical University, Guangzhou 510515, China.
** Corresponding author.
E-mail addresses: zlulu70@126.com (Z. Lu), zlilyzh@126.com (Z. Lin).
[1] These authors contributed equally to this work.

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Herein, in the present study, a thermosensitive hydrogel that was developed in our group and explored the therapeutic effects of this thermo-sensitive hydrogel encapsulated with BMSCs for the treatment of a full-thickness wound in a mouse model.

2. Materials and methods

2.1. Materials

N, N′-bis (acryloyl) cystamine, N-Isopropylacrylamide (NIPAM), Agmatine sulfate salt (AS), 1-(2-Aminoethyl)-piperozine (AEPZ), *N*,*N*,*N*′,*N*′-Tetramethylethanediamine (TEMED), Ammonium persulfate (APS), Lithium hydroxide (LiOH) and dithiothreitol (DTT), all the chemicals were purchased from Sigma-Aldrich. Bromodeoxyuridine (BrdU) (Sigma), α-smooth muscle actin (α-SMA) (Boster), keratin 1 (K1) (Abcam) and keratin 6 (K6) (Covance). CCK-8 proliferation assay kit was purchased from Fisher Scientific. DMEM medium, fetal bovine serum (FBS). glutamine and penicillin-streptomycin were ordered from Invitrogen. Cell culture plate was ordered from Corning. The basic-FGF and TGF-β1 ELISA kit were purchased from Shanghai Licheng Biological Technology co., LTD, China.

2.2. Synthesis of hyperbranched PAA crosslinker

MBA (234 mg, 1.52 mmol), Agmatine (138 mg, 0.6 mmol) and AEPZ (52 mg, 0.4 mmol) were dissolved in water/methanol (*v*/v = 5/1, total in 5 ml) while stirring. When the solution was clear, LiOH·H2O (25.2 mg, 0.6 mmol) was added to the solution. The mixture was then gently stirred and allowed to react at 40 °C in the dark for 72 h. After the reaction, the solvent was evaporated using a rotary-evaporator. The product was finally recovered by lyophilization and stored in-20 °C refrigerator for future use.

2.3. Characterization of hyperbranched PAA crosslinker

The hyperbranched PAA crosslinker was characterized by fourier transforms infrared spectroscopy (FTIR) and ^{1}H NMR, the detailed information as following. FTIR spectra were recorded with a Thermo Scientific IR100 FT-IR Spectrometer. PAA crosslinkers were freeze-dried in order to remove the residual chemicals. Then dried PAA crosslinkers were ground to powder, mixed with KBr, and compressed into pellets, then FTIR spectra were performed. In order to further characterize the hyperbranched PAA crosslinker, 1H NMR spectra (Fig. 1B) was recorded on an Advance 300 MHz spectrometer (Bruker). 8 mg of PAA crosslinker powders were dissolved in 800 μl of D2O. 1H NMR (ppm): *δ*, 1.5 (-N-CH2-CH2-CH2-CH2-NH-,s, 4 mH), 2.0–3.0(CO-CH2-CH2-N,m, 8(m + 2)H, -N2C4H8-CH2-CH2-N-,m, 8nH), 3.17(-N-CH2-CH2-,s, 2 mH), 4.53 and 4.62(-NH-CH2-CH-,s, 2 m + 2H), 5.77 (CH2 = CH-,t, 2H), 6.21(CH2 = CH-,s, 2H), 6.225 (-CH2 = CH,s, 2H).

2.4. Fabrication of thermo-sensitive hydrogel with hyperbranched PAA crosslinker

PAA crosslinked poly-NIPAM hydrogels were prepared at the concentration of 60 mg/ml with the ratio NIPAM: PAA = 5:1. Gels were fabricated in distill water by initiator APS (5.7 mg/ml) and catalyst TEMED (2.9 mg/ml) at room temperature for 30 min.

2.5. Characterization of physical properties

2.5.1. Swelling behavior test

The hydrogel was fabricated into a 2-mm-thick cylinder with a diameter of 10 mm and was then freeze-dried. The dry weight of this cylinder, W_0, was then measured. The dried cylinder was then placed in phosphate-buffered saline (PBS) (0.01 M; pH 7.4) at 37 °C and allowed to swell for 1.5 h, 3 h, 6 h, 9 h, 12 h or 24 h. The wet weight of the cylinder, Wt, was measured at each time point. The swelling ratios were calculated using equation (Eq.) [1]. For this test, $n = 6$, and the measurement was repeated 3 times [24].

$$\text{Swelling ratio (\%)} = (Wt - Wo)/Wo \times 100\% \quad (1)$$

2.5.2. In vitro degradation performance test

The hydrogel was fabricated into a 2-mm-thick cylinder with a diameter of 10 mm. After it was freeze-dried, the hydrogel cylinder was evenly divided into 4 parts. The dry weight of each of the 4 parts, Wd, was measured. Each of the 4 parts was then separately immersed into PBS (0.01 M; pH 7.4) containing 1 mol/ml DTT at 37 °C. The 4 parts were then harvested, one each on the 8, 16, 24 and 32 days. The remainder of each harvested part was first rinsed 3 times with distilled water at 37 °C and was then freeze-dried, after which the weight of the remaining part of hydrogel cylinder, Ww, was determined. The degradation rates were calculated using Eq. (2) [25]. For this test, $n = 6$, and the measurement was repeated 3 times.

$$\text{Degradation rate (\%)} = (Wd - Ww)/Wd \times 100\% \quad (2)$$

2.5.3. Hydrogel toxicity analysis

In accordance with International Organization for Standardization (ISO) 10993-5, extracts were prepared using 1 ml of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) for every 0.75 cm^2 of hydrogel. The hydrogel was laid flat in a 6-cm petri dish and UV sterilized for 30 min. DMEM was then added to the proportions described above, and the hydrogel was placed in a 37 °C incubator for 5 days for extraction. The hydrogels and extracts were then collected into 50 ml centrifuge tubes and centrifuged at 7000 rpm for 10 min. The supernatant was collected and filter sterilized as 100% hydrogel extracts. DMEM was used to dilute the 100% extracts to prepare 50% extracts. The toxicity of the hydrogel was evaluated using the CCK-8 proliferation assay kit [26]. First, one hundred milliliter L929 fibroblasts suspension (5×10^3 cells) were seeded in each well of 96-well plate and cultured for 24 h, after which the culture medium was removed. The 50% and 100% hydrogel extraction media were then added to the wells respectively. After the fibroblasts were cultured in different conditioned media for 1 day, 2 days or 3 days, 10 μL of CCK-8 was added into each well, and the fibroblasts were further incubated for 3 h. The absorption values at 450 nm were determined using an enzyme mark instrument (BIO-RAD). The relative proliferation rates were calculated using Eq. (3). There were 6 replicate wells in each group, and each measurement was repeated 3 times.

$$\text{Relative proliferation rate (\%)} = (\text{the sample})/(\text{the negative control}) \times 100\% \quad (3)$$

2.6. Culture and encapsulation of BMSCs

One healthy 8- to 12-week-old male C57BL/6 mice were purchased from the Laboratory Animal Centre of Southern Medical University. Briefly, bone marrow was obtained as in our previous study and then centrifuged at 1500 rpm for 5 min. The supernatant was discarded, and the precipitate was re-suspended in DMEM medium plus with 10% FBS. 1% glutamine and 1%penicillin-streptomycin, transferred into a petri dish, and cultured in an incubator at 37 °C and 5% CO2. The medium was replaced every 2 days, as the cells were subcultured once cell fusion reached 90% [27].

Fourth-generation BMSCs were trypsinized in 0.25% trypsin and centrifuged. Afterwards, the BMSCs were resuspended in saline and counted (cell concentration: 1 × 106 cells/ml). The BMSCs were again centrifuged, and 100 μl hydrogel was added to the centrifuged BMSCs. The hydrogel and the BMSCs were mixed to homogeneity using a pipette tip [28].