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ORIGINAL ARTICLE

Effect of the hyaluronic acid-poloxamer hydrogel on skin-wound healing: in vitro and in vivo studies

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Abstract

Background: Recent research into skin injury and wound healing has focused mainly on post-trauma hemostasis, infection prevention, dermal regeneration and angiogenesis. However, less attention has been paid to air permeability and moisture loss prevention which also play important roles in injury healing.

Methods: In the present work, we prepared a hyaluronic acid-poloxamer (HA-POL) hydrogel and tested the therapeutic effect of the hydrogel on skin-wound healing.

Results: The HA-POL hydrogel transformed from sol to gel at 30°C, close to body temperature, and had stable moisturizing properties. HA-POL hydrogel promoted skin-wound healing and increased protein accumulation in the wound area. HA-POL hydrogel allowed greater air permeability than Band-aid, a typical wound covering. Results from transwell assays showed that the HA-POL hydrogel effectively isolated skin-wounds from bacterial invasion.

Conclusion: This work demonstrates the advantages of using HA-POL gel materials in the treatment of cutaneous wounds.

KEYWORDS

HA-POL hydrogel, hyaluronic acid, poloxamer 407, skin-wound healing

1 | INTRODUCTION

Skin-wound healing is critical for successful outcomes of surgical and emergency medical procedures.[1-6] Skin wounds can be created by surgery, scalds, burns, physical trauma or animal bites.[7-10] Recent research related to skin-wound healing has focused mainly on four aspects: (a) post-injury hemostasis and promotion of coagulation;[11] (b) infection prevention during skin repair;[12] (c) fibroblast differentiation and neovascularization;[13,14] (d) granulation tissue regeneration.[15,16] The first two aspects, coagulation and infection prevention, apply to early skin-wound healing.[17] Growth factors promote subsequent germinal layer cell division and neovascularization,[18-20] and various types of nano-material such as gold and silver nanoparticles have been used to promote granulation tissue differentiation.[21-25] However, several issues around skin-wound healing are not fully resolved: (a) hemostatic and anti-infective materials are often ineffective at promoting wound healing;[26] (b) growth factors can induce hyperblastosis or tumorigenesis;[27,28] (c) although remarkable effects of nano-materials have been

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reported, gold or silver nanoparticles may remain in scar tissue for a long time.[29,30] Therefore, there is an urgent need to explore safer and more effective treatment strategies.

Hyaluronic acid (HA) is a nitrogen-containing mucopolysaccharide composed of disaccharide units of D-glucuronic acid-*N*-acetylglucosamine and is a major component of the extra-cellular matrix.[31,32] It is an important industrial material and medical/pharmaceutical excipient, and has been widely used in skin care products and tissue engineering.[33-35] However, as an aid to skin healing HA has several defects: (a) it has been characterized as a moisturizer with low viscosity;[36] (b) a single application of HA does not make the wound breathable.[37] The aim of this study was to improve the effect of HA on wound healing. We prepared and tested a HA-poloxamer (HA-POL) hydrogel and found that this new material can effectively promote skin-wound healing by enhancing protein accumulation in the wound area, increasing air permeability and preventing bacterial invasion.

2 | METHODS

2.1 | Agents and animals

Hyaluronic acid (HA) and Poloxamer 407 (POL) were purchased from Furuida Corporation, Shandong Province, China. Sprague-Dawley (SD) rats were purchased from Sibeifu Corporation, Beijing, China. Isoflurane (inhalation anesthetic) was purchased from RWD Life Science Co., Shenzhen, Guangdong Province, China. Basic fibroblast growth factor (bFGF) was purchased from ProTeck Corporation, USA. Pathology test kits (HE or Sirius Red staining) were purchased from Zhongshan JInqiao Corporation, Beijing, China. Transwell plates (Costar 3422®) were purchased from Corning Coporation, USA. *Escherichia coli* DH5α was cultured in our lab. Use of the experimental mice and all protocols were approved by the Institutional Animal Care and Use Committee of Fifth Medical Center, General Hospital of Chinese PLA. The project ethics review approval number is IACUC-2017-008.

2.2 | Preparation of HA-POL hydrogel

To prepare the hydrogel, 1 g of 1.2 million Da molecular weight HA, 4 g of 0.12 million Da molecular weight HA and 5 g of poloxamer 407 (POL) were weighed and dissolved by stirring in an ice bath, and the volume was made up to 100 ml with double-distilled H_2O. The resulting HA-POL gel was maintained at 4°C. The apparent viscosity (η) of the hydrogel was determined using a coaxial cylindrical rheometer (Cat# TC-502, Brookfield Corporation, USA) with the data processed by a workstation (Cat# Rheocale V3.0 Bld46, Brookfield Corporation, USA).

2.3 | Full-thickness wound healing experiments in rats

Adult male SD rats, with body weights of 180-220 g, were fed and housed in clean cages maintained at 22-25°C. At the start of the experiment, twenty rats were anesthetized by intraperitoneal injection of 10% (w/v) chloral hydrate (0.5 ml per animal). The dorsal skin was shaved and cleaned with an iodophor (0.2% w/v). The dorsal skin was then surgically operated to excise two full-thickness skin patches (diameter 2 cm^2) on each side of the upper backs of each animal. The left-side wound was chosen as the control and treated with an injection of physiological saline. The right-side wound was treated with drugs. The rats were randomly divided into two therapeutic groups (n = 10 in each group): one group of animals was treated with HA-POL hydrogel and the other group was treated with bFGF (100 AU/cm^2), which was chosen as a positive control. Both agents were applied directly by smearing on the skin wounds once per day. Photographs of skin wounds were taken at the indicated time points.

At the different time points, the two rats were sacrificed and full-thickness skin samples including intact skin adjacent to the wound, the wound margin and epithelialized wounds were collected and prepared as tissue slices. Slices were then examined using HE (hematoxylin and eosin)[38] and Sirius Red staining.[39]

2.4 | Biochemical analysis

At the time of sacrifice, granulation tissue from rat skin wounds was collected. The collected tissue was washed with physiological saline, and the protein and hydroxyproline (Hyp) concentrations in the granulation tissue were quantified according to the method of Li et al (2015), using, respectively, a Coomassie brilliant blue protein determination kit and a hydroxyproline kit.[23]

2.5 | Gas barrier test

Hydrogel films were prepared and the thickness of films was examined. Band-aid adhesive bandages of different thicknesses were used as controls. A moisture permeability analyzer (Cat# Permatran-W3/61, Mocon Corporation, Illinois, USA) was used to examine the water vapor transmission rate (WVTR, g/m^2/day) of hydrogel.[40] An oxygen permeability analyzer (Cat# 8001, Mocon Corporation, Illinois, USA) was used to examine the oxygen transmission rate (OTR, ccm/m^2/day) of hydrogel.[41] Test temperature (*T*) and relative humidity (RH) conditions were *T* = 25°C, RH = 0 for OTR measurement and *T* = 25°C, RH = 50% for WVTR measurement.

2.6 | Transwell assays

Transwell assays were performed to test bacterial migration across a solvent control, HA gel or HA-POL gel, which were added to the undersides of the upper chambers of the assay vessels.[42,43] *E. coli* was cultured to 1 O.D./ml and diluted with LB medium (1:1000). Diluted *E. coli* (100 μl) was added to the upper chambers. The LB medium in the lower wells was cultured to analyze the migration of *E. coli* from the chambers to the wells. Migrated *E. coli* were quantified using a

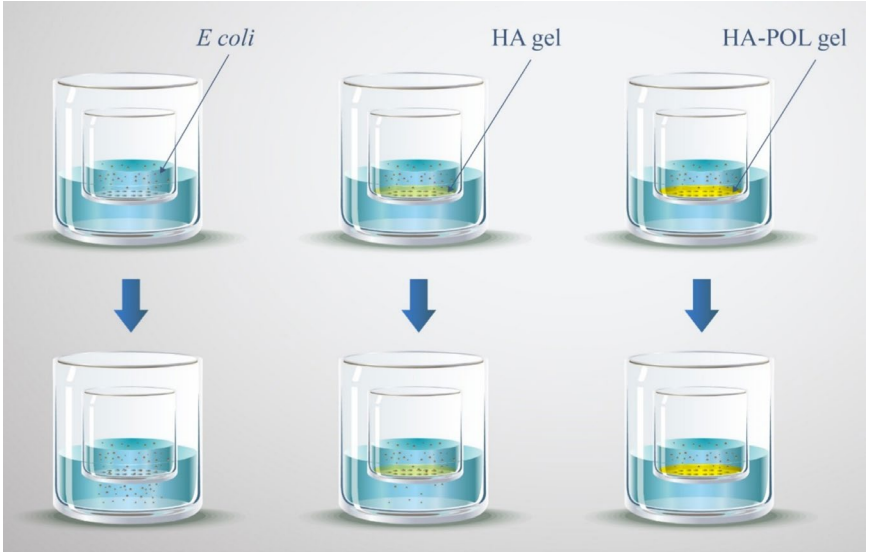


FIGURE 1 The schema chart of transwell experiments. Chambers of transwell plates were coated by control, HA or HA-POL hydrogel. The migration of *E. coli* could be prevented by HA gel or HA-POL gel

multifunctional microplate reader set for O.D. 600 nm. Figure 1 is a schematic diagram of the transwell experiments.

3 | RESULTS

3.1 | HA-POL hydrogel temperature sensitivity

As shown in Figure 2, the apparent viscosities of HA-POL or POL hydrogels increased with temperature. The HA-POL hydrogel transformed from sol to gel at 30°C, whereas the POL hydrogel transformed from sol to gel at 37°C. The results indicated that HA-POL hydrogel forms films at a temperature close to body temperature.

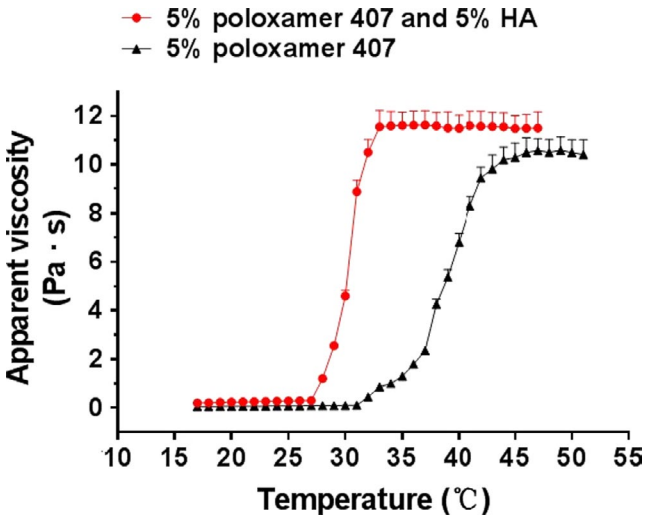


FIGURE 2 Temperature-viscosity Curve of hydrogel. Apparent viscosity (η) of HA-POL hydrogel or POL 407 was examined at different temperatures (t)

3.2 | HA-POL hydrogel promotes skin-wound healing

First, we examined the overall effect of HA-POL hydrogel on skin-wound healing. Figure 3A shows the skin-wound healing process in rats receiving control (saline), bFGF (basic fibroblast growth factor) or HA-POL hydrogel treatments at different time points. By day 14, the skin wounds of the rats in the HA-POL hydrogel group were almost completely healed whereas the wounds of the control and bFGF groups were still visible.

Next, we examined the protein and hydroxyproline (Hyp) content of the wounds to reveal the extent of protein accumulation during the healing process. As shown in Figure 3B and C, the total protein and Hyp content in the HA-POL-treated group was higher than in the control or bFGF groups.

We next performed pathological staining assays, using HE and Sirius Red staining, to further investigate the effect of the hydrogel. As shown in Figure 4, granulation tissue formation and angiogenesis were identified by HE staining (Figure 4A), whereas fibroblast accumulation and collagen deposition were examined by Sirius Red staining assays (Figure 4B). HA-POL hydrogel promoted fibroblast accumulation, granulation tissue formation, angiogenesis and collagen deposition more effectively than control and bFGF groups.

3.3 | HA-POL hydrogel effectively protects skin wounds from *E. coli*

Next, transwell experiments were performed to examine the migration of bacteria across the gel. *E. coli* was added to the upper chambers, the undersurfaces of which were coated with control saline solution, HA hydrogel or HA-POL hydrogel. As shown in Figures 1 and 5, *E. coli* migrated from the chambers to the wells below when the chambers were coated with saline and this migration was attenuated by HA hydrogel for 2-3 days and by HA-POL gel for over 5 days. These data show that HA-POL hydrogel effectively protected the wounds from bacterial infection.

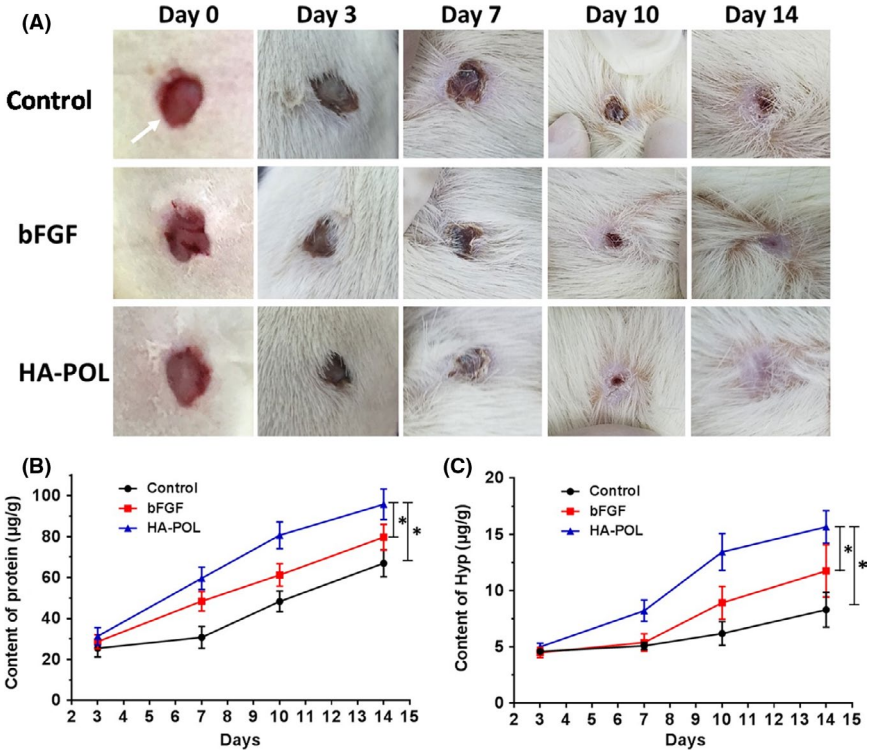


FIGURE 3 Effect of agents on the healing of full-thickness skin-wounds in rats' model. A, Rats were surgically operated to form full-thickness wounds. Skin-wounds were treated by control, bFGF or HA-POL hydrogel. Representative photographs during wound-healing process at indicated time points were shown. B, The contents of total-protein in granulation tissues or Hyp of skin-wounds was shown as mean±SD. $^{*}P < 0.05$

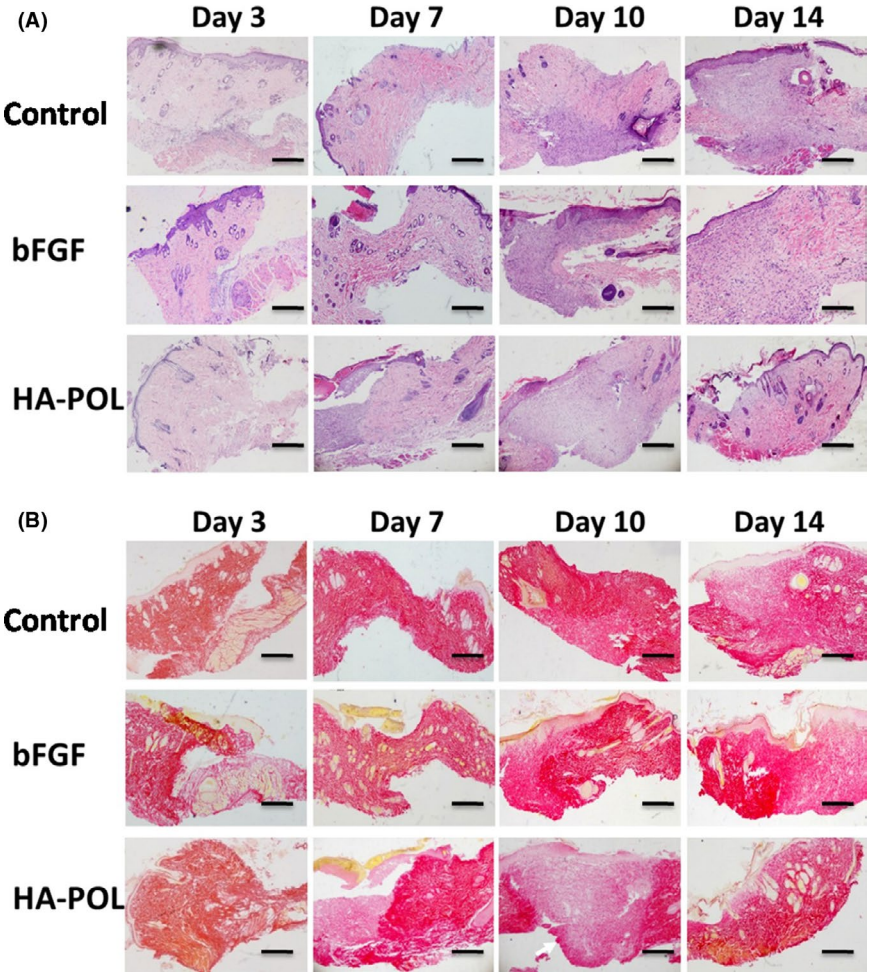
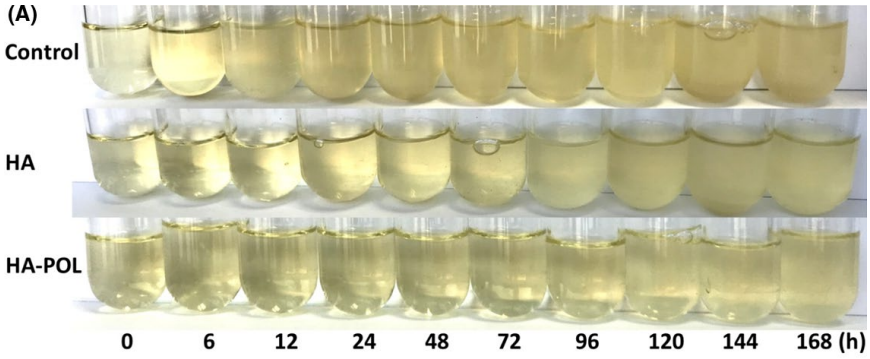


FIGURE 4 Effect of agents on the healing of full-thickness skin-wounds in rats' model. A, Rats were surgically operated to form full-thickness wounds. Skin-wounds were treated by control, bFGF or HA-POL hydrogel. Tissues specimens were analyzed by pathological assays. Representative photographs from HE staining (A) or Sirius Red staining (B) was shown. Scale bar =100 μm



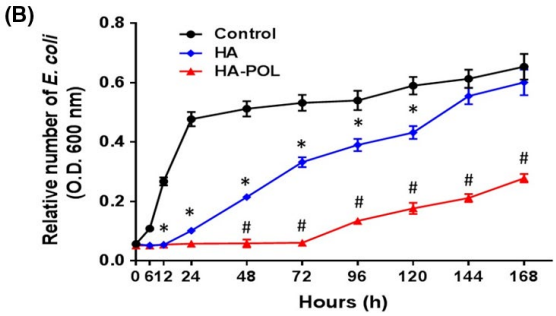
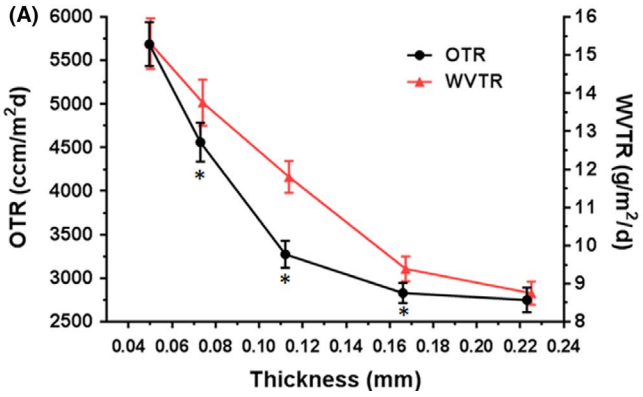


FIGURE 5 Effect of agents on preventing infection of *E. coli* in a transwell model. Chambers of transwell plates were coated by control, HA or HA-POL hydrogel. *E. coli* was added in chambers and the migration of *E. coli* was identified by examining the *E. coli* in wells. Medium in wells was shaking culture in LB for 5-6 hours. The photograph (A) and O.D. 600nm (B) was shown. $^{*}P < 0.05$ versus control with HA; $^{\#}P < 0.05$ versus HA with HA-POL



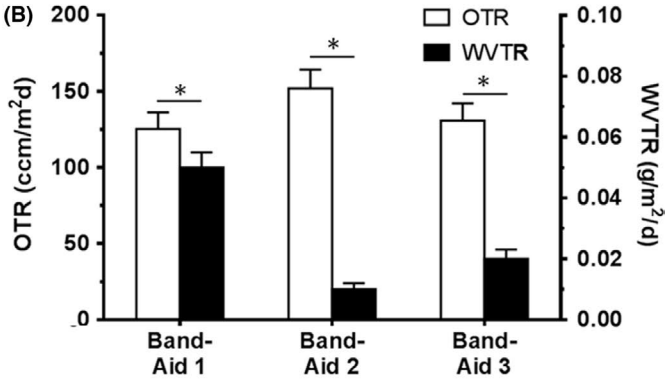


FIGURE 6 WVTR and OTR curve of films formed by HA-POL gel with different thickness. A, HA-POL hydrogel was used to form a film. The WVTR and OTR of films formed by HA-POL hydrogel with different thickness was examined. B, The WVTR and OTR of three kinds of Band-Aid with different thickness (Band-Aid 1: 1.5mm; Band-Aid 2: 0.8mm; and Band-Aid 3: 1.0mm) was examined as control. $^{*}P < 0.05$

3.4 | HA-POL hydrogel has good moisture absorbing and air permeability capacities

The moisture absorption and air permeability of HA-POL hydrogel was examined. As shown in Figure 6, unlike Band-Aid adhesive bandages, which can completely block oxygen and water vapor transmission, HA-POL hydrogel showed good moisture absorbing and air permeability capacities. This finding indicated that HA-POL hydrogel may be a promising material for promoting wound healing.

4 | DISCUSSION

Skin-wound healing is a complex process involving multiple steps: stratum corneum cells proliferate to form a scab and then fibroblasts generate new granulation tissue to fill the wound scars and wrap necrosis tissues.[44,45] Final differentiation of the hair follicles and hair structure are essential for wound repair.[46,47] Among current treatments, Band-aid adhesive bandages are used to prevent hemostasis, ointments containing antibiotics are used to prevented infections, and growth factors, eg bFGF, are used to promote fibroblasts proliferation.[48,49] Recently, nanoparticles have been used as novel treatments in skin-wound healing: silver nanoparticles are used as anti-infection agents, while gold nanoparticles are used to stimulate fibroblast proliferation and collagen secretion.[50] However, gold and silver nanoparticles may deposit sub-dermally for long periods of time and be difficult to excrete,[51] and the cost of these promising materials may be a major obstacle to wide clinical and out-patient use.

HA has been widely used in cosmetic R & D, plastic surgery and other surgical procedures due to its good moisturizing capacity. However, HA hydrogel does not form a film, which may be critical for infection protection. We prepared a gel by combining HA with Poloxamer 407, a poloxamer that is safe to use, and examined its properties in skin-wound healing. In our experiments, we found that HA-POL hydrogel has better film-forming properties. HA-POL hydrogel is much better at promoting skin-wound healing than growth factor bFGF, and may protect skin-wounds from bacterial infection. The water vapor and oxygen transmission capacities of HA-POL hydrogel are better than Band-aid; oxygen transmission measurements indicated that HA-POL hydrogel has good air permeability capacity and could be a promising material for wound healing.

Our results suggest that HA-POL hydrogel offers a number of potential improvements in skin-wound healing: (a) HA-POL is biodegradable and thus avoids problems associated with non-degradable treatments; (b) its effective moisturizing and air permeability capacities help to prevent infection and protect wounds, potentially avoiding the use of antibiotics; (c) HA-POL hydrogel treatment of skin trauma enhances tissue repair, potentially avoiding the need for growth factor treatment. Taken together, our findings demonstrate that HA-POL hydrogel is a promising material for wound healing.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

All listed authors meet the requirements for authorship. JH, RSL and FF conceived and designed the experiments; XJL and AML performed the experiments and wrote the main manuscript text. SHW and YTC performed the animal experiments; QTJ and RCY performed the molecular biology experiments and analyzed the data. ZJW performed the pathological experiments. All authors have read and approved the manuscript.

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