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Healing effect of carboxymethyl chitosan-plantamajoside hydrogel on burn wound skin

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ABSTRACT

Background: It is known that hydrogels based on carboxymethyl chitosan (CMCS) have properties controling microbial growth, reducing inflammatory cell infiltration, and promoting collagen deposition. Plantamajoside (PMS), a natural Chinese herbal medicine with biological activity, has the properties of reducing inflammation, anti-oxidation, and promoting wound healing. However, the effects of carboxymethyl chitosan/plantamajoside hydrogel on partial thickness burn wounds remain unclear.

Methods: The healing effect of carboxymethyl chitosan/plantamajoside hydrogel was evaluated by in vitro cell viability assay, cell migration assay, and further evaluated in a rat model of partial-thickness burn wounds.

Results: The hydrogels were highly porous with a pore size of about 250 μm, and these pores were interconnected. After adding plantamajoside, a dense microstructure was further formed. The hydrogels containing 0.25% plantamajoside significantly increased the viability and migration of L929 cells ($P < 0.05$). Carboxymethyl chitosan/plantamajoside hydrogel significantly improved wound healing, granulation tissue proliferation and re-epithelialization, and promoted collagen deposition ($P < 0.05$). Carboxymethyl chitosan/plantamajoside hydrogel also significantly decreased IL (interleukin)-1β, IL-6 and TNF-α expression, and increased IL-10 expression ($P < 0.05$). Furthermore, carboxymethyl chitosan/plantamajoside hydrogel significantly promoted the expression levels of VEGF, CD31, α-SMA (α-smooth muscle actin) and collagen III, and reduced the expression level of collagen I ($P < 0.05$). Our data suggest that carboxymethyl chitosan/plantamajoside hydrogel promotes burn wound healing by accelerating angiogenesis and collagen deposition and reducing the inflammatory response.

1. Introduction

Burn injury wound exudates, usually including plasma, proteins, microbes and the products of their metabolism, antibodies, red and white blood cells, and platelets, provide

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sufficient high nutrients for the growth and reproduction of microorganisms, which increase the wound infection rate and subsequent strong inflammatory response [1]. It is well known that repair of tissue damage is a complex and orderly biological process, including hemostasis, inflammatory response, cell proliferation and tissue remodeling [2,3]. Inflammation is a crucial stage of scald wound healing, which is mainly characterized by continuous infiltration of neutrophils, macrophages and lymphocytes [4]. Scald is only one form of burn injury. There are others, and once the skin is injured, the healing process is the same including the formation of exudates commonly mistaken for infection. Although considerable progress has been made in the care and treatment of burn wounds, local infection is still one of the main risks of death. Therefore, it is of great significance to develop an ideal wound repair dressing that is non-toxic, strong fluid absorption, good biocompatibility and strong anti-inflammatory activity.

Carboxymethyl chitosan prepared by carboxymethylation of chitosan has high solubility and stability in neutral and alkaline solutions. Due to its non-toxic, biodegradable and biocompatible properties, carboxymethyl chitosan-related products have recently been widely used in wound dressings and drug carriers [5]. Chitosan hydrogels can not only control microbial growth, but also promote cell proliferation, collagen fibrosis and formation of hyaluronic acid [6,7]. There are abundant amino groups on the surface of chitosan, which is the key to the antibacterial properties of chitosan. Chitosan promotes the growth of cells related to wound healing, such as the growth of normal fibroblasts, epithelial cells and keratinocytes, and macrophages to produce active factors that contribute to wound healing; Chitosan promotes microvascular regeneration of skin wound tissue and improves blood circulation, thus accelerating wound healing. The molecular structure of chitosan dressing is similar to that of mucopolysaccharide matrix in cell stroma and has good cytocompatibility, which provides a favorable environment for the growth of skin cells and is beneficial to wound healing. In addition, the chemotaxis of carboxymethyl chitosan on neutrophils and macrophages helps prevent wound infections in the initial stage of wound repair, and helps promote the formation of granulation tissue and the regeneration of epidermal cells during the healing period of wound repair [8]. Furthermore, carboxymethyl chitosan fabricated sponge, membrane and hydrogel dressings has a porous sponge structure with the best water absorption, air permeability and hemostatic properties, and carboxymethyl chitosan sponge can promote the proliferation of human skin fibroblasts and greatly increase the expression levels of TGF-β1 (transforming growth factor-β1) and α-SMA(α-smooth muscle actin) [9]. These studies suggest that carboxymethyl chitosan hydrogel can be used as a potential dressing to accelerate scald wound healing.

Herba plantaginis, a traditional Chinese herbal medicine, is widely distributed in East Asia, especially in China. Plantamajoside ($C_{29}H_{36}O_{16}$), a kind of phenylpropanoside compound, is one of the main bioactive components in Herba plantaginis [10]. Plantamajoside has a variety of biological activities, such as antibacterial, antioxidant, antiinflammatory, promoting wound healing, anti-virus and antitumor activities [11,12]. Long-term and high-dose oral plantamajoside is safe in rats. No adverse reactions were observed in rats with oral plantamajoside more than 2000 mg/kg for 13 weeks [13]. In addition to its inhibitory effects on lipopolysaccharide-induced lung injury, plantamajoside was also reported to reduce the production of inflammatory cytokines by inhibiting the TLR4-mediated NF-κB and MAPK signaling pathways [12]. However, the healing effect of carboxymethyl chitosan/plantamajoside hydrogel on second-degree burn wound remains unclear. Therefore, this study prepared the carboxymethyl chitosan/plantamajoside hydrogel, and evaluated its effect on cell viability and migration by cell viability and wound healing experiments, respectively. Furthermore, the healing effect of the carboxymethyl chitosan/plantamajoside hydrogel on rats with deep second-degree burn skin wound was evaluated.

2. Materials and methods

2.1. Materials and animals

Carboxymethyl chitosan (CMCS, carboxylation > 80%) and glucuronic acid deltalactone (GDL) were obtained from Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China). Plantamajoside (99.4% purity) was provided by Best-reagent company (Chengdu, China).

A total of 54 male SD rats were used in this study, from the Benxi Changsheng Biotechnology Co., Ltd (Benxi, China). All rats were kept in an environment of 20-24 °C with a humidity of 55% + 5% and a light/dark cycle of 12/12 h. During the period, ad libitum access to water and food was provided to the rats. All rats were equally divided into control group (n = 18) carboxymethyl chitosan hydrogel (n = 18), and carboxymethyl chitosan/plantamajoside hydrogel group (n = 18). The study protocol was approved by the Institutional Animal Care and Use Committee of General Hospital of Northern Theater Command.

2.2. Preparation of carboxymethyl chitosan/plantamajoside hydrogels

Carboxymethyl chitosan/plantamajoside hydrogel was provided by Dr. Zhao Yan (Institute of Metal Research, Chinese Academy of Sciences, China). First, three stock solutions were prepared. For Solution A, carboxymethyl chitosan was fully mixed with sodium alginate (total 0.25 g, 1:1 w/w), and 10 ml deionized water was added. Solution B was prepared by dissolved 0.04 g glucuronic acid delta-lactone (GDL) in 1 ml of deionized water. Solution B is freshly prepared every time it is used. Solution C was prepared by dissolving 0.05 g PMS in 800 μl absolute ethanol. Afterwards, Solution A, B and C were filtered through a 0.2 μm polytetrafluoroethylene filter. After adding 0.1 ml of Solution B to 1 ml of solution A, the carboxymethyl chitosan hydrogel can be formed in about 10 min. For the preparation of carboxymethyl chitosan/plantamajoside hydrogel, after adding 0.1 ml of Solution B and 0.1 ml of Solution C were into 1 ml of Solution A, vortex for 2 min to mix homogeneously. The hydrogel was formed in about ten minutes.

2.3. Rheological characterization

The rheological characterization of carboxymethyl chitosan/plantamajoside hydrogel was performed as described [15]. Briefly, in order to make the dynamic storage modulus (G′) and the loss modulus (G″) independent of the strain amplitude, the strain amplitude was further optimized to ensure the measurement in the linear viscoelastic region. During the frequency sweep, 1 Hz oscillatory frequency and 0.001% strain (within the linear viscoelastic region) were used to avoid interferences of the measurement with the gelling process. Regarding the isothermal frequency dependence, both dynamic storage modulus (G′) and the loss modulus (G″) were measured at 37 °C and a constant strain of 0.001% in the frequency range of 0.1–10 Hz.

2.4. Morphology of hydrogels

The morphology of carboxymethyl chitosan and carboxymethyl chitosan/plantamajoside hydrogels was assessed using scanning electron microscopy (SEM, inspect F50, FEI). The freeze-dried hydrogels were prepared into some circular disks (about 3-5 mm diameter), and then covered with a gold layer for SEM observation.

2.5. Cell viability assay

The human skin fibroblasts (L929 cells) were cultured in DMEM medium containing 10% fetal bovine serum (FBS). The control, carboxymethyl chitosan-0.05% plantamajoside, carboxymethyl chitosan-0.1% plantamajoside, and carboxymethyl chitosan-0.25% plantamajoside hydrogels were dissolved in PBS, and then added to the 96-well plates. The hydrogels were cut to be able to cover all the L929 cells on the culture plate. 20 μL of MTT solution (5 mg/mL) was added, and incubated at 37 °C for 4 h, then 100 μL of dimethyl sulfoxide (DMSO) was added, shaking for 10 min to dissolve the MTT formazan crystals.

2.6. Cell migration assay

A cell migration assay was done as described [16]. Briefly, fibroblasts (L929, 5×10^5 cells/mL) were cultured in 24-well plates. When L929 cells grew to 100% confluence, linear scratch wounds were created on the back of the wells with a sterile 200 μL pipette tip, and then cells were cultured in DMEM medium containing 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C. After 0, 24, 48 and 72 h, the scratched areas were photographed at 100× magnification using a Leica DMI3000B microscope (OlympusBX41, Tokyo, Japan). Moreover, Image-Pro Plus v6.0 analysis software was used to analyze all images.

2.7. Skin burn wound

A partial-thickness burn skin wound was performed as described [17]. Briefly, 10% Chloral hydrate (4 ml/kg) was injected intraperitoneally to anesthetize the rats. The ultra-high temperature controller (YLS-5Q, Beijing, China) was set at 100 °C, and the metal punch (2.0 cm^2) was pressed tightly on the rat skin for 8 s. Only one wound was made on the back skin. The partial-thickness burn skin wound was confirmed by histopathological observation. After 1 h, the dead skin was surgically removed by employing a device (JW-180 sander) with hard plastic sanding. When we use a tool to remove crusts, stop when necrotic tissue is treated to expose fresh tissue (blood exudation). Partial-thickness wound depth was confirmed by the histopathological observation. The wounds were covered with the carboxymethyl chitosan and carboxymethyl chitosan/plantamajoside hydrogels, respectively, and then treated using thermoplastic polyurethane (TPU) (Shanghai Yuanye Biotechnology Co., Ltd, China) dressings. While the wounds were covered using the TPU dressing in the control group. All rats were treated once a day for 5 consecutive days.

2.8. Wound healing rate

After the rats were anesthetized with isoflurane, the wound exudate, scab and degree of wound healing were observed and recorded. Meanwhile, the area of wound healing was measured using the Image-Pro Plus v6.0 image analysis software. The wound area was photographed and the wound closure at each time point was calculated based on the following formula: = (Area on day 0−Open area on day 10)/Area on day 0 × 100%.

2.9. Histological analysis

Skin samples collected on days 5, 10 and 16 after scalding was fixed in 10% neutral-buffered formalin, and then the paraffin-embedded skin samples were subsequently cut into 3-5 μm thick slices, and stained with hematoxylin and eosin (H&E) and Masson's trichrome, respectively. From each scanned Masson's trichrome stained section, 5 random fields of view were manually selected and imaged at an objective lens magnification of 20x. Collagen deposition was analyzed using Image-Pro Plus v6.0 analysis software.

2.10. Western blotting

Western blotting in this study was performed as described [18]. Briefly, protein extraction kit (Fdbio, Science) was used to extract total protein from fresh skin samples, and then the concentration of protein in skin tissue was determined by BCA kit (Fdbio, Science). A 10% polyacrylamide gel was used to isolate the protein, transfer it to a nitrocellulose membrane, and then the procedure of western blotting was performed. All antibodies used in this study were as follows: Collagen III(sc-271249) and CD31 (sc-376764, 1:1000, Santa Cruz, USA); iNOS (ab178945), CollagenI(ab270993)and α-SMA (ab245222) (1:500, Abcam, UK); VEGFR (ab2349), iNOS (ab178945) (1:1500, Abcam, UK), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:4000, Cell Signaling Technology, Boston, MA, USA).

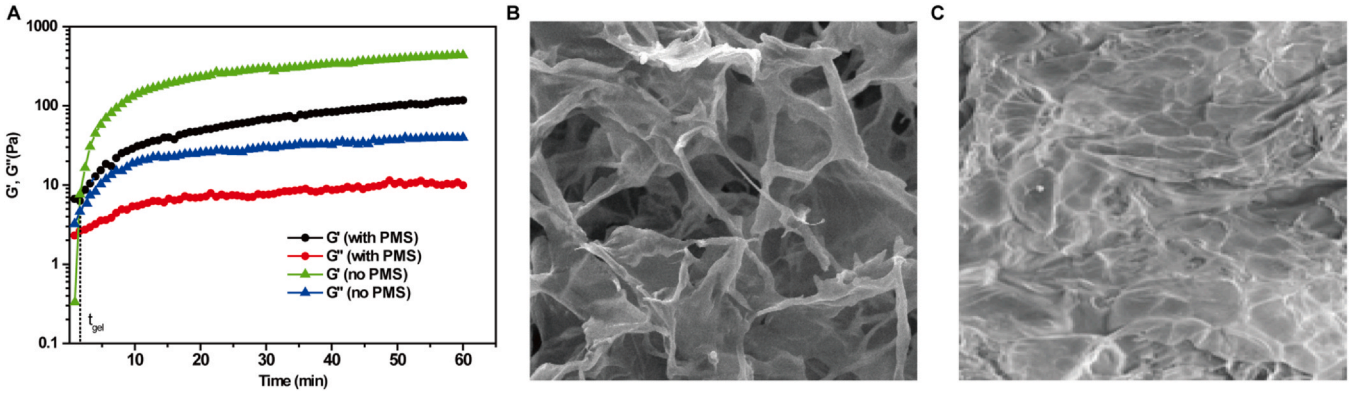

Fig. 1 – Characterization and microstructures of CMCS/alginate-PMS hydrogels. (A) The rheological characterization of CMCS/alginate and CMCS/alginate-PMS hydrogels. (B,C) The morphology of CMCS/alginate (B) and CMCS/alginate-PMS hydrogels (C) was assessed using scanning electron microscopy.

2.11. *Statistical analysis*

Data were expressed as the means ± standard error (SE), and analyzed using one-way ANOVA with Tukey post hoc test. A two-sided $P < 0.05$ was considered statistically significant.

3. Results

3.1. *Characterization and microstructures of carboxymethyl chitosan/plantamajoside hydrogels*

The dynamic storage modulus G′ and the loss modulus G″ were measured as a function of time to study the gelling kinetics of the carboxymethyl chitosan/plantamajoside hydrogels. In the two hydrogels with and without plantamajoside, G′ and G″ both dramatically increased with time (Fig. 1A). In the hydrogel without plantamajoside, G′ exceeded G″ in about two minutes. In contrast, in the hydrogel with plantamajoside, G′ exceeded G″ in the initial phase of the measurement. The G′ and G″ of the hydrogel without plantamajoside were, respectively, higher than the value of hydrogel with plantamajoside, revealing that plantamajoside may affect the loss of mechanical properties (ca 5-fold in G' and G''). Moreover, the hydrogels were highly porous with a pore size of about 250 μm, and these pores were interconnected (Fig. 1B). After adding plantamajoside, a dense microstructure was further formed. Due to the limited solubility of plantamajoside in water, the addition of ethanol-dissolved plantamajoside to the hydrogel caused a certain degree of aggregation in the carboxymethyl chitosan/plantamajoside hydrogel.

3.2. *The effects of carboxymethyl chitosan/plantamajoside hydrogel on cell viability*

As shown in Fig. 2, higher cell viability was observed in carboxymethyl chitosan introduced with 0.05%, 0.1% and 0.25 plantamajoside, respectively. Among them, carboxymethyl chitosan -0.25% plantamajoside showed the highest cell viability, suggesting that the addition of plantamajoside increased the promoting effect of carboxymethyl chitosan on the viability of L929 cells.

3.3. *Effects of carboxymethyl chitosan/plantamajoside hydrogel on cell migration*

After 12 h of carboxymethyl chitosan/plantamajoside hydrogel treatment, significant cell migration was observed at the edge of scratch, and the widest gap was seen in the control group (Fig. 3). After 24 h, carboxymethyl chitosan/plantamajoside hydrogel further narrowed the gap. After 36 h, the gap was completely disappeared in the carboxymethyl chitosan-0.25% plantamajoside-hydrogel group. The cell migration rate of carboxymethyl chitosan/plantamajoside-0.05% plantamajoside ($P = 0.045$), chitosan/plantamajoside-0.1% plantamajoside($P = 0.038$) and carboxymethyl chitosan-0.25% plantamajoside($P = 0.021$) groups was significantly increased compared with the control group. The highest migration rate occurred in the carboxymethyl chitosan-0.25% plantamajoside group. The plantamajoside significantly improved gap closure with respect to plantamajoside-free conditions. These data indicate that carboxymethyl chitosan/plantamajoside hydrogel promotes the migration of L929 cells.

3.4. *Carboxymethyl chitosan/plantamajoside hydrogel promoted the wound healing rate*

As shown in Fig. 4A, on first day after burn injury, the burn wounds in the three groups had a nearly round and edematous structure with softened and blanched surfaces. After the necrotic tissue was completely removed, the wound showed obvious bleeding and exudation. On day 5th day after burn injury, edema appeared on the wound surface of the control group, accompanied by tissue fluid exudation and purulent substance secretion. However, obvious scab was observed in the carboxymethyl chitosan hydrogel and the carboxymethyl chitosan/plantamajoside hydrogel groups. On the 10th day after burn, obvious scab was observed in the control group, while the scab in the carboxymethyl chitosan hydrogel group and carboxymethyl chitosan/plantamajoside hydrogel group

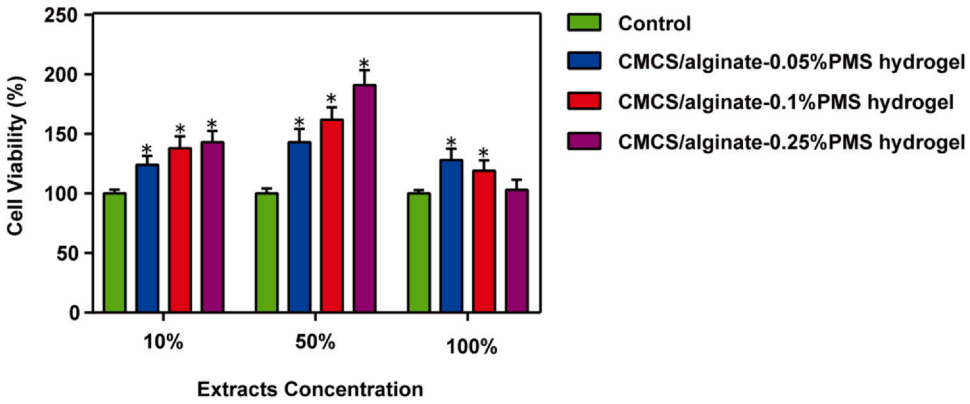

Fig. 2 – The effects of CMCS/alginate-PMS hydrogel at different concentrations on L929 cell viability. The human skin fibroblasts (L929 cells) were cultured in DMEM medium, and cell viability was determined by MTT method as described in the Materials and methods. Data were represented as the mean ± SEM. *$p < 0.05$, *vs.* control group.

began to fall off. On day 16th day after burn, there were still a small amount of reddish and swelling scab attachments in the control group and the carboxymethyl chitosan hydrogel group. However, the scabs in the carboxymethyl chitosan/plantamajoside hydrogel group almost completely fell off, and obvious granulation tissues were found. Image J was further used to quantify the rate of wound healing in the three groups. As shown in Fig. 4B, the carboxymethyl chitosan/plantamajoside hydrogel group showed the best overall improvement in the healing of burn wound among the three groups. On the 5th and 10th day after burn, the carboxymethyl chitosan/plantamajoside hydrogel group showed a higher wound healing rate compared with the control group and the carboxymethyl chitosan hydrogel group ($P < 0.05$). On the 16th day after burn, the wound healing rate in the carboxymethyl chitosan/plantamajoside hydrogel group was higher than that in the control group and the carboxymethyl chitosan hydrogel group, although it did not reach a significant statistical difference($P > 0.05$).

3.5. *Carboxymethyl chitosan/plantamajoside hydrogel accelerated wound healing in rats*

On the 5th day after injury, the epidermis of rats in each group was severely damaged (Fig. 5). There were many necrotic hair follicle structures and inflammatory cell infiltration in the dermis, and the dermis matrix was loose. Compared with the control group, carboxymethyl chitosan group and carboxymethyl chitosan/plantamajoside hydrogel significantly reduced inflammatory cell infiltration. Meanwhile, granulation tissues with a few collagen deposition and new capillaries were observed in the carboxymethyl chitosan/plantamajoside hydrogel group. In the control group and the carboxymethyl chitosan hydrogel group, the necrotic tissue was significantly reduced on the 10th day after burn. The granulation tissue increased significantly, and the necrotic tissues formed obvious scabs. In contrast, in the carboxymethyl chitosan/plantamajoside hydrogel group, the granulation tissue gradually filled the tissue defect, the epidermal cells began to crawl, and finally the scabs fell off. On the 16th day after burn injury, almost intact epithelium was seen in the carboxymethyl chitosan/plantamajoside hydrogel group. Collagen in the dermis layer was neatly arranged without obvious edema and inflammatory cell aggregation. However, the dermal layer was still collapsed, with dermal edema and marked inflammatory cell infiltration in the control and carboxymethyl chitosan hydrogel groups.

3.6. *Carboxymethyl chitosan/plantamajoside hydrogel promoted collagen deposition*

Tissue remodeling is the final stage of wound repair, which is characterized by progressive increase of collagen content and reorganization. Since Masson's trichrome staining is a classical staining of collagen fibers, the scalded skin of each group in this study was stained with Masson staining to detect the collagen content. As shown in Fig. 6A, on the10th and 16th day after burn injury, the collagen content of the control group and carboxymethyl chitosan hydrogel group were low, and the collagen distribution was sparse and disorder. In contrast, the collagen fibers of the carboxymethyl chitosan/plantamajoside hydrogel group were neatly arranged and uniformly distributed. Meanwhile, the collagen content was quantified by Image-pro plus software. As shown in Fig. 6B, as compared to the control and the carboxymethyl chitosan hydrogel groups, the collagen content was significantly increased in the carboxymethyl chitosan/plantamajoside hydrogel group ($P < 0.05$). Moreover, as compared to the control group and CMCS/alginate hydrogel group, collagen III expression level in the carboxymethyl chitosan/plantamajoside hydrogel group was significantly increased on the 10th and 16th day after burn injury (Fig. 6C).

3.7. *Carboxymethyl chitosan/plantamajoside hydrogel reduced the expression level of proinflammatory factors*

It is known that inflammation plays a crucial role in the healing of burn wounds. In order to explore the inflammatory effect of carboxymethyl chitosan/plantamajoside hydrogel on burn wounds, the expression of TNF-α, IL-1β, IL-6, and IL-10 in skin samples was detected using western blotting. Compared with the control group and carboxymethyl

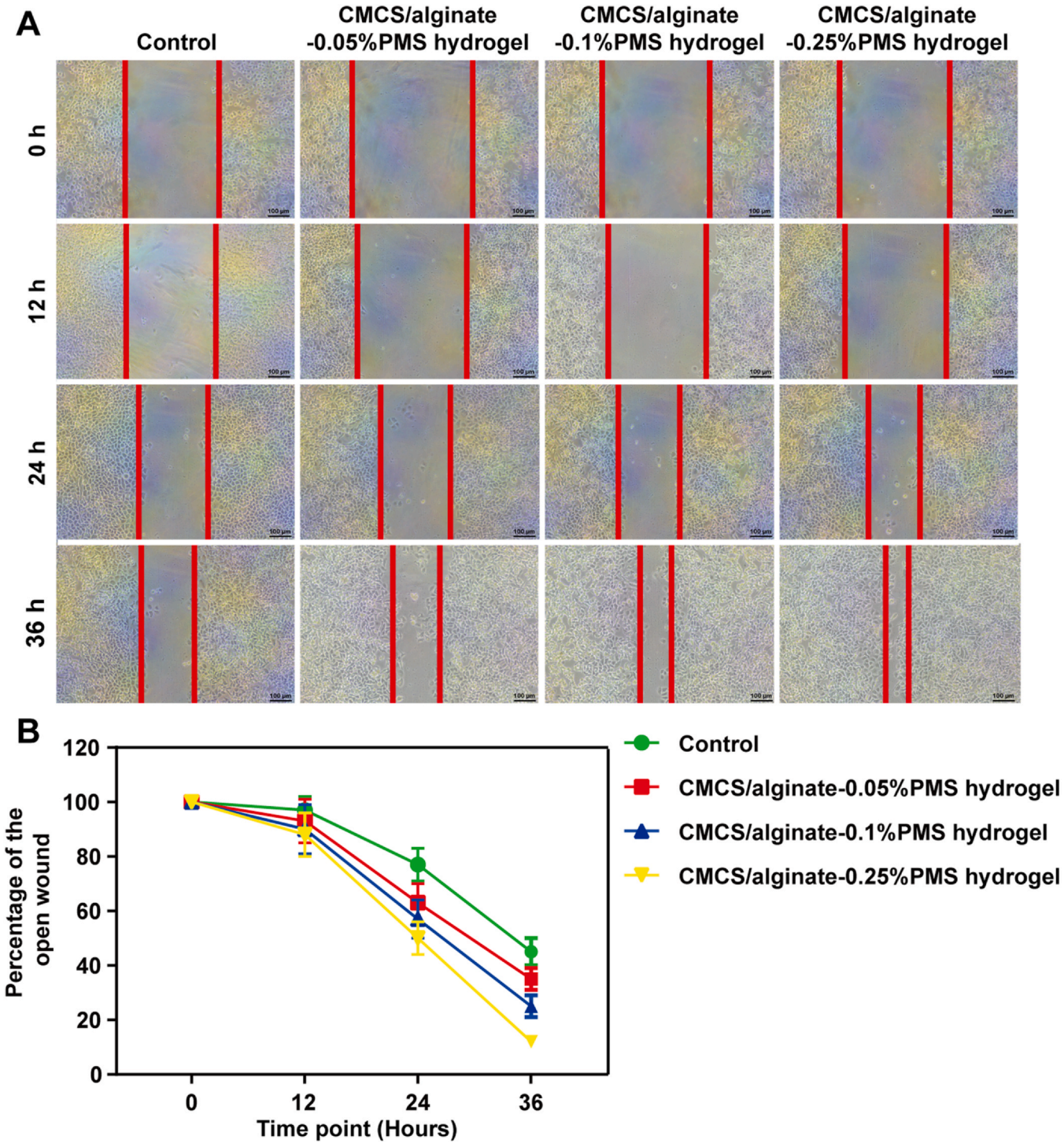

Fig. 3 – The effects of CMCS/alginate-PMS hydrogel on cell migration. Cell migration assay was used to investigate the effect of different concentration CMCS/alginate-PMS hydrogel on migration of L929 cell. Data were represented as the mean ± SEM. *p < 0.05, vs. control group.

chitosan hydrogel group, the expression of TNF-α, IL-1β and IL-6 in the carboxymethyl chitosan/plantamajoside hydrogel group was significantly decreased on the 5th, 10th, and 16th day after burn injury (Fig. 7). As compared to the control group and CMCS/alginate hydrogel group, the expression levels of IL-10 in the carboxymethyl chitosan/plantamajoside hydrogel group was significantly increased on the 5th, 10th and 16th day after burn injury.

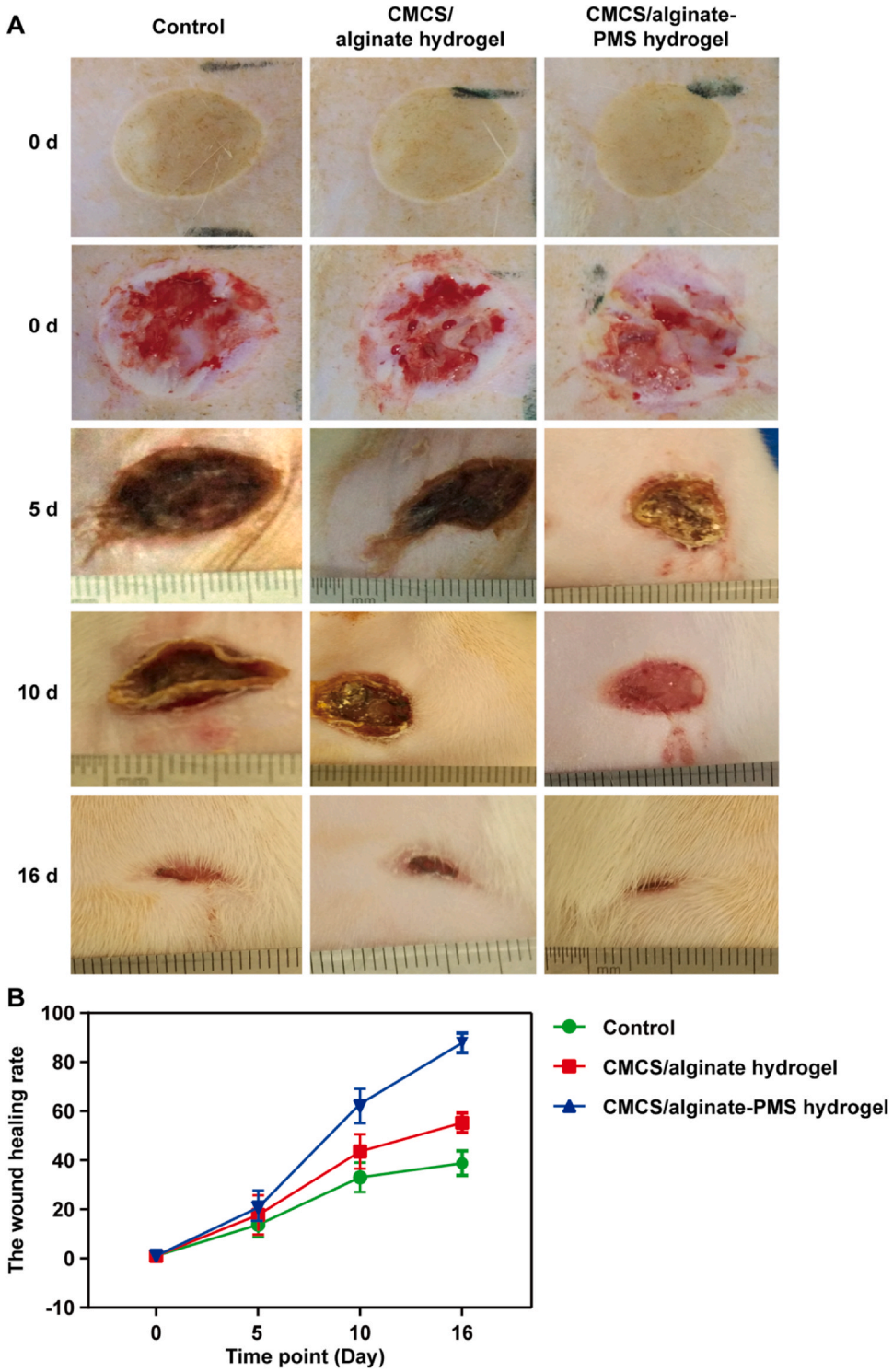

Fig. 4 – CMCS/alginate-PMS hydrogel promoted the wound healing rate. (A) Representative pictures of wound healing in each group on day 0, 5, 10 and 16 after burn. (B) The rate of scald wound healing. Data were represented as the mean ± SEM of values obtained from 18 rats in each group. *$p < 0.05$, *vs*. control and CMCS/alginate hydrogel groups.

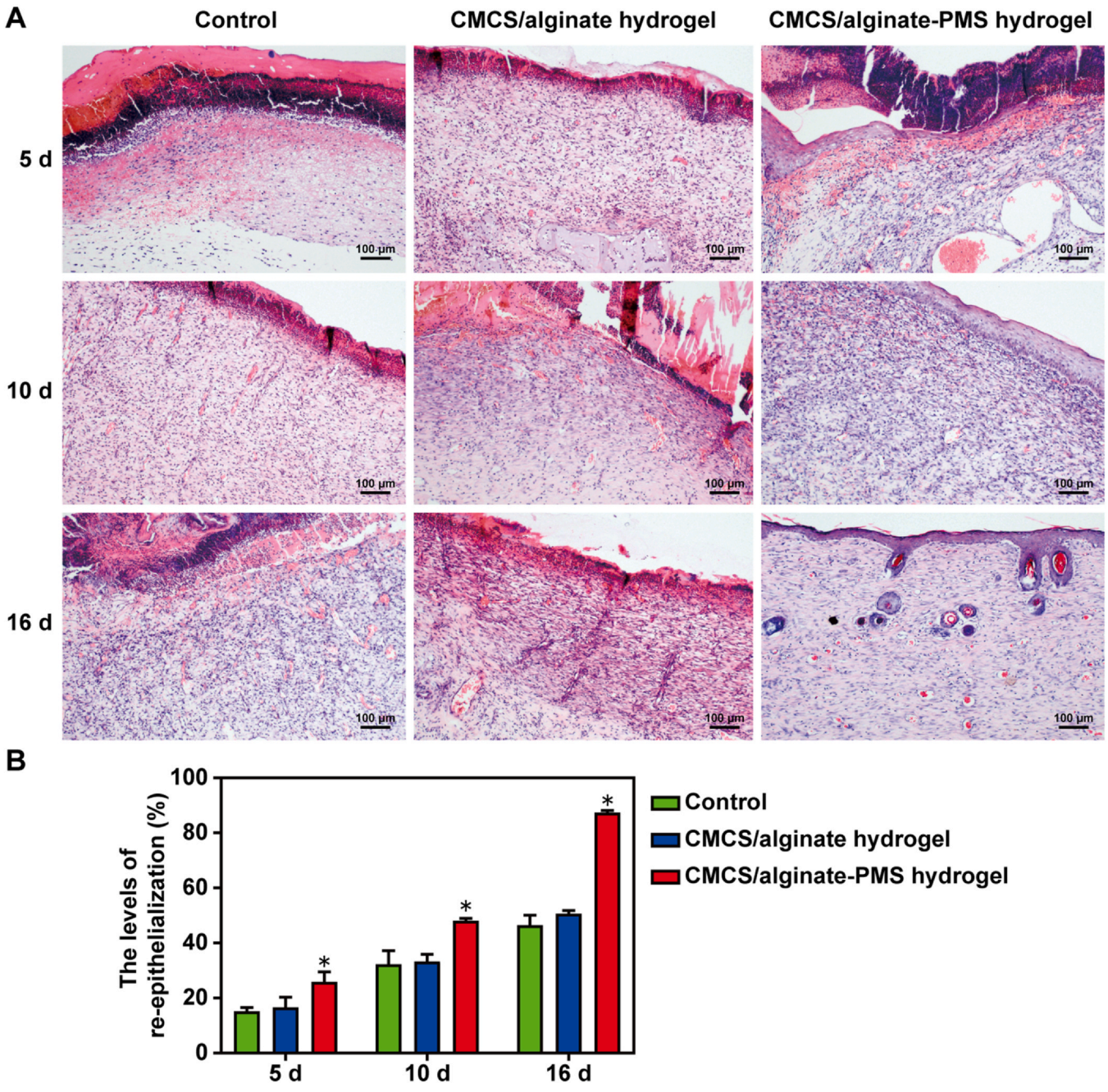

Fig. 5 – CMCS/alginate-PMS hydrogel promoted the process of wound healing in rats. (A) Representative HE staining pictures in the control, CMCS/alginate and CMCS/alginate-PMS hydrogel groups (bar = 100 μm). (B) Re-epithelialization in burn wound healing. Data were represented as the mean ± SEM of values obtained from 18 rats in each group.*$p < 0.05$, vs. control and CMCS/alginate hydrogel groups.

3.8. *Carboxymethyl chitosan/plantamajoside hydrogel promoted angiogenesis and collagen expression*

The process of wound healing involves the formation of blood vessels in subcutaneous tissue. On the 5th, 10th, and 16th day after burn injury, the expression of VEGFR and CD31 in skin tissues, both of which are biomarkers of vascular endothelial cells, were detected by western blot analysis. As shown in Fig. 8, as compared to the control group and the carboxymethyl chitosan hydrogel group, the expression of VEGF and CD31 in the carboxymethyl chitosan/plantamajoside hydrogel group increased significantly on 5th and 10th day after burn injury. Furthermore, carboxymethyl chitosan/plantamajoside hydrogel significantly increased the expression levels of α-SMA and collagen III, and reduced the expression of collagen I on the 5th, 10th, and 16th day after burn injury. One notable exception was that compared with the control group, the expression of inducible nitric oxide

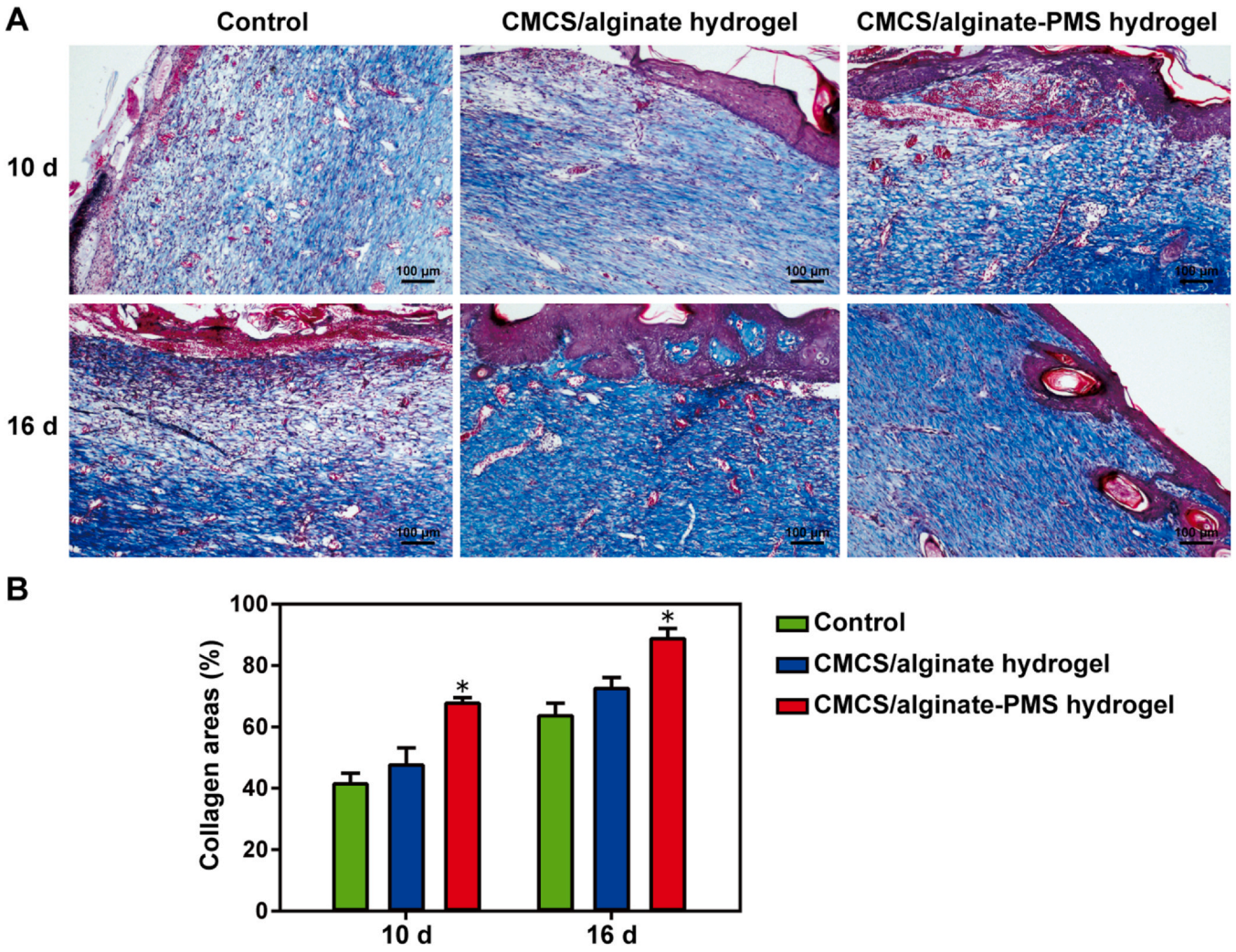

Fig. 6 – CMCS/alginate-PMS hydrogel promoted collagen deposition (A) Representative pictures of Masson's staining (bar = 100 μm). (B) Area of collagen deposition in burn wound healing. Data were represented as the mean ± SEM of values obtained from 18 rats in each group. *$p < 0.05$, *vs.* control and CMCS/alginate hydrogel groups.

synthase (iNOS) significantly increased in the carboxymethyl chitosan hydrogel group and carboxymethyl chitosan/plantamajoside hydrogel group on the 5th to 16th day after burn injury.

4. Discussion

It is reported that carboxymethyl chitosan-based hydrogels can prevent wound infection, reduce inflammation, and promote fibroblast proliferation and collagen deposition [19,20]. Plantamajoside is a natural Chinese herbal medicine that can reduce inflammation and promote cell proliferation and angiogenesis [21,22]. The results of this study indicated that plantamajoside significantly affected the biological activities and microstructure of carboxymethyl chitosan hydrogel. The hydrogels containing 0.5% plantamajoside can significantly increase the viability and migration ability of L929 cells. carboxymethyl chitosan/plantamajoside hydrogel not only significantly increased wound closure, but also markedly increased granulation tissue proliferation, re-epithelialization, and collagen deposition. Carboxymethyl chitosan/plantamajoside hydrogel also significantly reduced the expression of IL-1β, IL-6 and TNF-α, and increased the expression of IL-10. Furthermore, carboxymethyl chitosan/plantamajoside hydrogel significantly promoted the expression levels of VEGF, CD31 and collagen III. Our data suggest that carboxymethyl chitosan/plantamajoside hydrogel can accelerate the healing of scald wound in rats by promoting angiogenesis and collagen deposition and reducing inflammation.

The process of wound repair involves many cell types such as macrophages and fibroblasts [23]. Hydrogel is widely regarded as a good wound dressing due to its high-water retention, high permeability and easy to detach from the surface of wound [24]. Due to its good biocompatibility, nontoxicity and promotion of cell proliferation in vitro, carboxymethyl chitosan hydrogel is a very attractive substitute to chitosan in hydrogel preparation [25]. It is reported that carboxymethyl chitosan /collagen and chondroitin sulfate

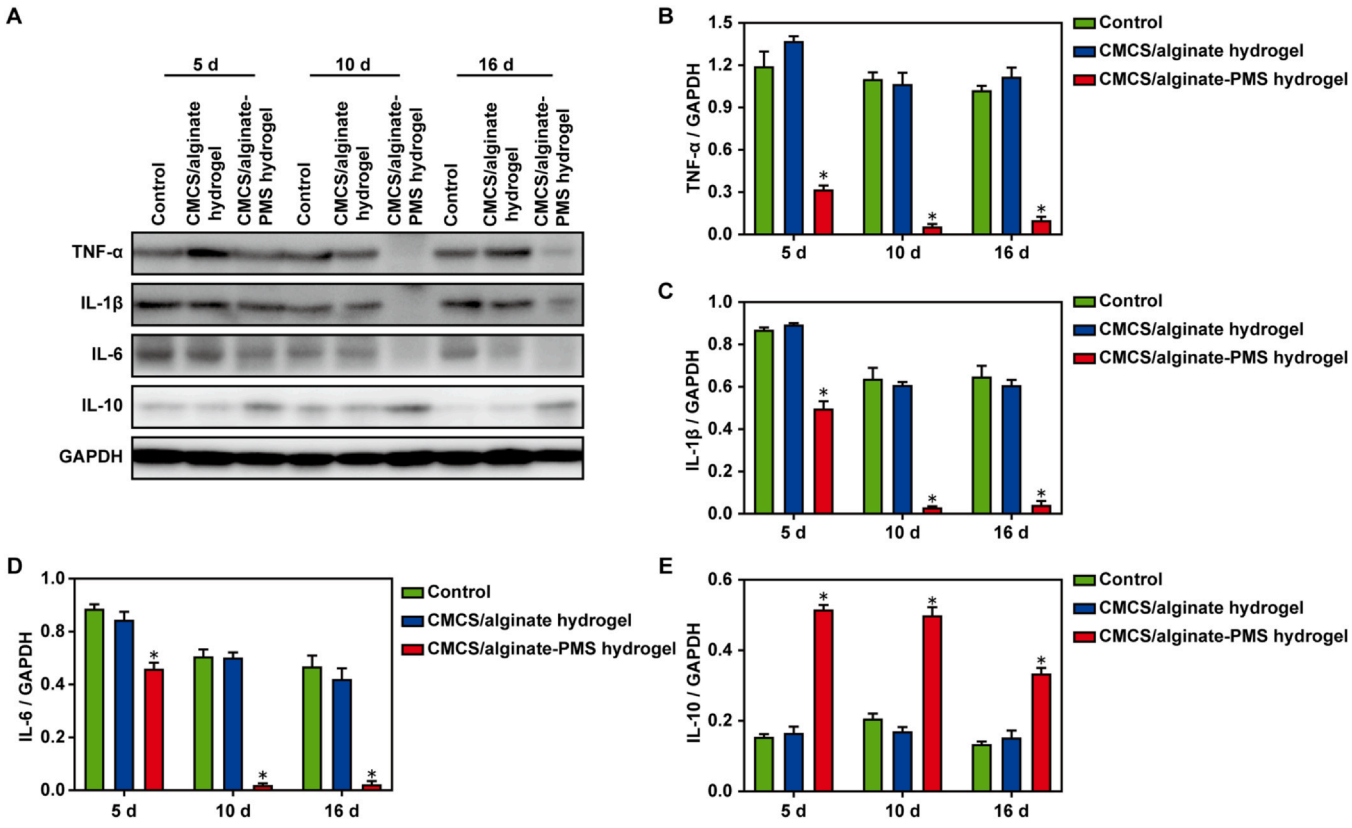

Fig. 7 – CMCS/alginate-PMS hydrogel reduced proinflammatory factors expression. (A) Western blot images of TNF-α, IL-1β, IL-6, IL-10 and GAPDH. (B–E) The expression levels of TNF-α, IL-1β, IL-6, and IL-10 with normalized to internal control GAPDH. Data were represented as the mean ± SEM of values obtained from 18 rats in each group. *$p < 0.05$, *vs.* control and CMCS/alginate hydrogel groups.

composite scaffolds significantly promoted the migration, proliferation and secretion of cytokines of fibroblasts, thereby contributing to the healing of full-thickness skin wounds in rats [26]. The results of this study showed that plantamajoside significantly affected the microstructure and biological activity of carboxymethyl chitosan hydrogels, and the hydrogels containing 0.25% plantamajoside significantly increased the viability and migration of L929 cells, and plantamajoside also significantly improved gap closure with respect to PMS-free conditions. Meanwhile, carboxymethyl chitosan/plantamajoside hydrogel can not only significantly promote wound closure and granulation tissue proliferation, but also accelerate epithelial reformation and collagen deposition. In the early stage of wound healing, fibroblasts appeared in the wound and began collagen synthesis. In the late stage of healing, the thickness and density of collagen fibers increased. The carboxymethyl chitosan/plantamajoside hydrogel significantly promotes collagen fiber synthesis in the early stage. Ethanol- and water-based plantamajoside extracts have been reported to significantly stimulated the wound healing in porcine skin, and both have best effects at a plantamajoside concentration of 1.0 mg/mL [27]. In addition, plantamajoside can attenuate $CoCl_2$-induced migration and invasion in HepG2 cells by inhibiting the epithelial-mesenchymal transition (EMT) process, and inhibit the malignancy of HepG2 cells under hypoxic condition by inhibiting the of expression HIF-1α [28]. Genc, et al. further reported that the isolated constituents from plantamajoside are potential wound healing agents by their remarkable inhibitory activities against collagenase, elastase and hyaluronidase [29]. One of the carboxymethyl chitosan and carboxymethyl chitosan/plantamajoside hydrogels contained an important quantity of ethanol, whereas the control without ethanol. Throughout the study, we did not set up a control group added with ethanol to exclude rule out the effect of ethanol in the PMS formulation, which is also one of the limitations of this study. The results of this study indicate that carboxymethyl chitosan/plantamajoside hydrogel can mediate the healing of scald wounds, and it should be used in the development of wound dressings in the future.

In the present study, our data showed that carboxymethyl chitosan/plantamajoside hydrogel significantly promoted angiogenesis, including increased expression of VEGFR and CD31 and reduced the expression levels of proinflammatory IL-6, TNF-α and IL-1β. As reported, the anti-inflammatory properties of plantamajoside can significantly inhibit hypoxia-induced migration and invasion of human cervical cancer cells by inhibiting the NF-κB and PI3K/Akt pathways [30]. The mechanism of plantamajoside may be partly by inhibiting the activation of PI3K/Akt signaling pathway, and then inhibiting the inflammation and NF-κB activation. A study by Liu et al. demonstrated that plantamajoside can

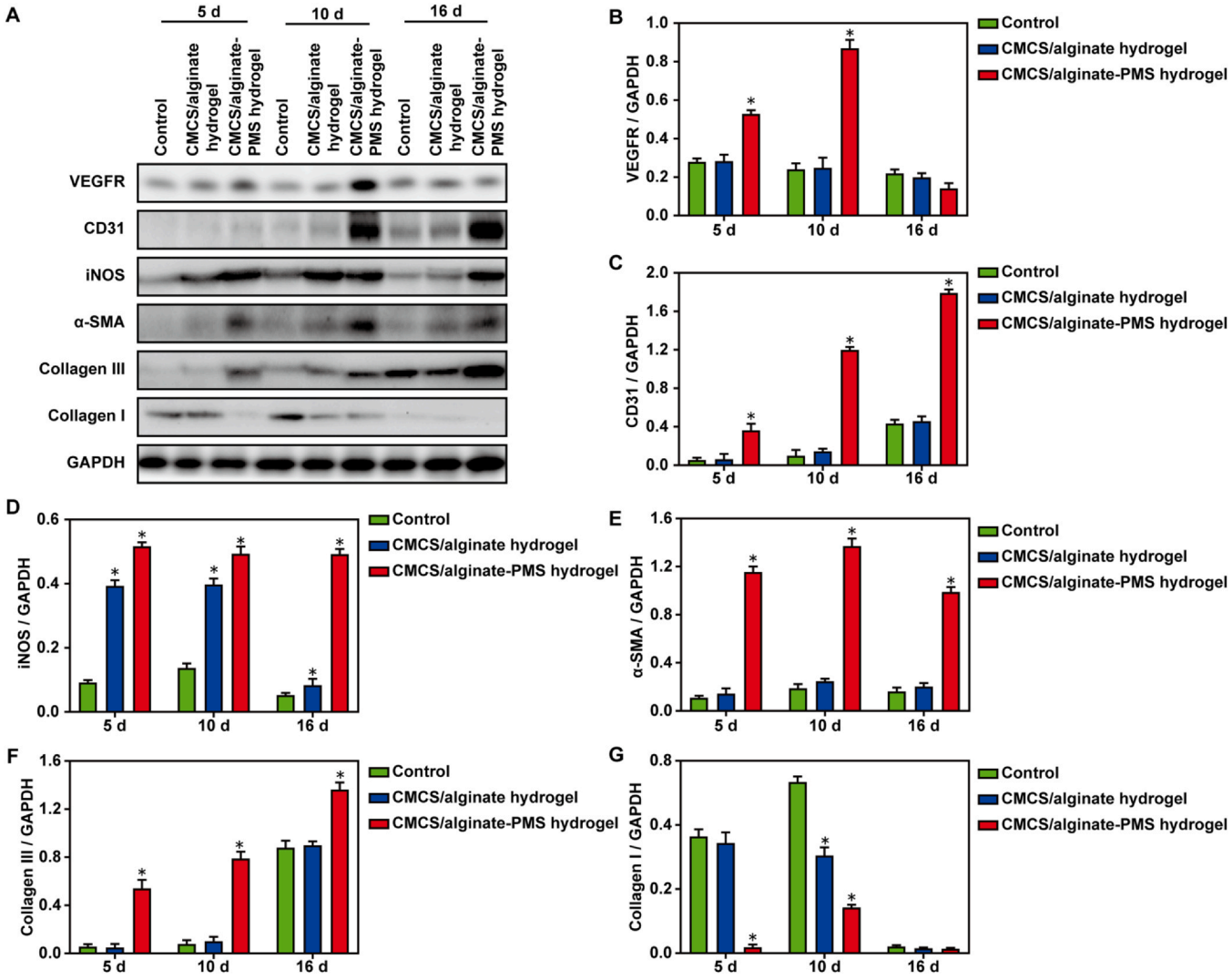

Fig. 8 – CMCS/alginate-PMS hydrogel promoted angiogenesis and collagen protein expression. (A) Western blot images of angiogenesis and collagen deposition protein. (B–D) The expression levels of VEGFR, CD31 and iNOS relative to GAPDH. (E-G) The expression levels of α-SMA, collagen III, and collagen I with normalized to internal control GAPDH. Data were represented as the mean ± SEM of values obtained from 18 rats in each group. *$p < 0.05$, *vs.* control and CMCS/alginate hydrogel groups.

significantly reduce the expression levels of PGE2, NO, IL-6 and IL-8 in human gingival fibroblasts (HGFs) stimulated by lipopolysaccharide, and decreased the phosphorylation levels of NF-κB p65, IκB, PI3K and AKT [31]. Furthermore, plantamajoside has been reported to inhibit LPS-induced EMT by inhibiting the NF-κB/IL-6 signaling cascade in esophageal squamous cell carcinoma (ESCC) cell lines [32]. Plantamajoside can also suppress oxidative stress, inflammation, and extracellular matrix accumulation by inactivating the Akt/NF-κB pathway, thereby alleviating high glucose-induced injury in HBZY-1 cells [33]. In the present study, carboxymethyl chitosan/plantamajoside hydrogel significantly promoted the expression levels of collagen III, α-SMA, and TGF-β1, and accelerated the process of wound healing. Contrary to our results, the study by Want et al. demonstrated that plantamajoside can reduce the increase of protein and mRNA levels of α-SMA and collagen type 1 α1 (Col1α1) in platelet-derived growth factor BB (PDGF-BB)-treated HSC-T6 cells [34]. It is likely that plantamajoside exhibits an anti-fibrotic effect in the liver by inhibiting hepatic stellate cell activation and survival. We speculate that plantamajoside may attenuate hepatocyte injury caused by PDGF-BB by inhibiting fiber deposition, while plantamajoside promotes rapid wound healing by promoting fiber proliferation and accelerating collagen deposition. The fundamental reason may be that plantamajoside initiates different repair modes according to different models to reduce the damage caused by adverse factors to the body. Rodent wound healing is generally by contraction rather than epithelialisation which is different from human wound healing. Because plantamajoside is difficult to dissolve in water, and can only be dissolved in ethanol solution. So it can not be directly applied to rat wounds. Therefore, plantamajoside alone group was not set up in this study. These findings support our results that

carboxymethyl chitosan/plantamajoside hydrogels can accelerate wound repair by promoting angiogenesis during the initial stage of wound healing and suppressing pro-inflammation during the late stage of wound healing.

In conclusion, our data demonstrate that carboxymethyl chitosan/plantamajoside hydrogel promoted the process of burn wounds healing through accelerating angiogenesis and collagen deposition, and reducing inflammatory response in rats.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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