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Wound healing with alginate/chitosan hydrogel containing hesperidin in rat model

Zohreh Bagher[a,1], Arian Ehterami[b,c,1], Mohammad Hossein Safdel[d], Hossein Khastar[e], Hossein Semiari[f], Azadeh Asefnejad[g], Seyed Mohammad Davachi[h], Mehdi Mirzaii[i], Majid Salehi[j,k,*]

[a] ENT and Head & Neck Research Center and Department, The Five Senses Institute, Hazrat Rasoul Akram Hospital, Iran University of Medical Sciences (IUMS), Tehran, Iran
[b] Skull Base Research Center, The Five Senses Institute, Hazrat Rasoul Akram Hospital, Iran University of Medical Sciences (IUMS), Tehran, Iran
[c] Department of Mechanical Engineering, Science and Research Branch, Islamic Azad University, Tehran, Iran
[d] Faculty of Tissue Engineering, Department of Biomedical Engineering, Science and Research Branch, Islamic Azad University, Tehran, Iran
[e] School of Medicine, Shahroud University of Medical Sciences, Shahroud, Iran
[f] Doctor of Dental Surgery, Faculty of Dentistry, Shahed University, Tehran, Iran
[g] Department of Biomedical Engineering, Science and Research Branch, Islamic Azad University, Tehran, Iran
[h] Department of Food Science, College of Agriculture and Life Sciences, Cornell University, Ithaca, NY, USA
[i] Department of Microbiology, School of Medicine, Shahroud University of Medical Sciences, Shahroud, Iran
[j] Department of Tissue Engineering, School of Medicine, Shahroud University of Medical Sciences, Shahroud, Iran
[k] Tissue Engineering and Stem Cells Research Center, Shahroud University of Medical Sciences, Shahroud, Iran

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ABSTRACT

Skin damages have always been considered as one of the most common physical injuries. Therefore, many researches have been conducted to find an efficient method for wound healing. Since hydrogels have suitable characteristics, they are widely used for this purpose. In this study, based on the high efficiency of alginate and chitosan hydrogels in the wound healing, different concentrations of hesperidin were loaded to alginate and chitosan hydrogels followed by evaluating their morphology, swelling properties, release, weight loss, hemo- and cytocompatibility, antibacterial and toxicity properties. Finally, the therapeutic function of the prepared hydrogels was evaluated in the full-thickness dermal wound in a rat model. Our results indicated that the hydrogels have appropriate porosity (91.2 ± 5.33%) with the interconnected pores. Biodegradability of the prepared hydrogel was confirmed with weight loss assessment (almost 80% after 14 days). Moreover, the time-kill assay showed the antibacterial properties of hydrogels, and MTT assay revealed the positive effect of hydrogels on cell proliferation, and they have no toxicity effect on cells. Also, the in vivo results indicated that the prepared hydrogels had better wound closure than the gauze-treated wound (the control group), and the highest wound closure percentage was observed for the alginate/chitosan/10% hesperidin group. All in all, this study shows that alginate/chitosan hydrogels loaded with 10% of hesperidin can be used to treat skin injuries in humans.

1. Introduction

Skin, as the most significant human organ, is able to maintain the skin integrity following injury; however, multiple factors can lead to interrupt the wound healing [1–3]. In the United States, it has been estimated that more than 6.5 million people suffer from chronic and non-healing wounds, the cost of which exceeds $25 billion per year [4]. Over the last two decades, several new dressings and drug therapies have been developed to improve normal wound healing [5,6]. Ideally, the wound dressing should prepare a moist environment, protect the wound from infection, and also contribute to accelerate the wound healing process [7]. Therefore, since hydrogels provide most of the

* Corresponding author. Department of Tissue Engineering, School of Medicine, Shahroud University of Medical Sciences, Shahroud, Iran.
E-mail addresses: Bagher.z@iums.ac.ir (Z. Bagher), arian.ehterami@srbiau.ac.ir (A. Ehterami), mh.safdel@yahoo.com (M.H. Safdel), h_khastar@yahoo.com (H. Khastar), hossein.semi@gmail.com (H. Semiari), asefnejad@srbiau.ac.ir (A. Asefnejad), Md946@cornell.edu (S.M. Davachi), mirzaii1386@gmail.com (M. Mirzaii), salehi.m@shmu.ac.ir (M. Salehi).
[1] These authors contributed equally to this work.

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desirable characteristics of an ideal dressing, they have been widely used for wound healing [8,9]. They are versatile, reducing the pain by promoting moisture and cooling the wound surface, and also, exhibit a wide range of mechanical properties [10,11]. They can be fabricated from naturally occurring materials, synthetic polymers, or their hybrids [12,13]. Using natural materials such as chitosan has many advantages due to its biocompatibility, biodegradability, and hemostatic feature but it has not enough flexibility leading to be easily broken into smaller pieces [14,15]. The poor mechanical properties of chitosan could be improved by addition of alginate, which also has the advantage of biodegradability, biocompatibility, ability to absorb large amounts of fluid, and as a polyanionic polymer, its carboxyl group can interact with the amino group of chitosan [16,17]. Although hydrogels can guide the healing process to heal the wounds, much attention is needed to stimulate the proper healing of dermal wounded.

Hesperidin, flavonoids present in fruits and vegetables, have widespread biological features such as anti-inflammatory, antibacterial, antioxidant, angiogenic effects, which indicates that hesperidin may be a therapeutical drug in skin diseases [18–20]. Several more recent developments in wound dressings have focused on the localized drug delivery of wound healing due to the unwanted adverse effects of systemic drug delivery, such as harmful side-effects into target tissues and less predictable drug delivery to the target tissue [21–23]. Due to their tunable physical properties such as swelling and degradability, hydrogels are capable of controlling the drug release rate making them a beneficial drug delivery system for clinical use [10,11]. A previous study showed alginate-chitosan hydrogel contributed to the acceleration of wound repair due to its intrinsic antimicrobial activity [24]. Given the beneficial effect of hesperidin to improve the synthesis of collagen, it prevents haemorrhages and infection, antioxidants, and anti-inflammatory effects, and thus, we hypothesize that the combination of hydrogel and hesperidin could have a synergetic effect to accelerate the wound healing [25,26]. To the best of our knowledge, the effect of topical application of hesperidin-loaded hydrogel has not been studied in wound healing studies yet. Therefore, in the present study, the effect of different doses of hesperidin-loaded hydrogel on the healing of skin injury in a rat model was evaluated.

2. Materials and methods

2.1. Materials

Sodium alginate (Pharmaceutical grade, molecular weight 120000), chitosan (Pharmaceutical grade medium molecular weight 162.16), hesperidin, penicillin, streptomycin, calcium chloride (CaCl2), and glutaraldehyde were all purchased from Sigma-Aldrich (St. Louis, USA). 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT), Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F12), and fetal bovine serum (FBS) were purchased from Gibco, BRL (Eggenstein, Germany).

2.2. Hydrogel preparation

To prepare alginate/chitosan/hesperidin (Alg/Chit/Hes) hydrogel, first sodium alginate (2% w/v) was dissolved in deionized water. Then chitosan (2% w/v) was dispersed in acetic acid (0.5% v/v) and gently stirred for 24 h at 40 °C to dissolve completely. The sodium alginate and chitosan solutions mixed with ratio of 2:1 v/v. Next, the various concentrations of hesperidin (0, 1, 10% weight of polymer Alg/Chit) was added to the Alg/Chit solution. Afterward, in order to initiate gelation, calcium chloride 50 mM ($CaCl_2$) and 10 μl glutaraldehyde with NaOH 1 M were added to crosslink the alginate and chitosan, respectively. Finally, the prepared solution was mixed and vortexed for 1 h.

2.3. Lyophilization

The prepared solutions were first frozen for 16 h at −80 °C. To prepare the porous hydrogels, the frozen gels were lyophilized using a freeze drier (Telstar, Terrassa, Spain) for 48 h at −54 °C.

2.4. Hydrogel characterization

2.4.1. Morphological properties

The sample was freeze-dried, and the dry sample cut in the diameter of 7 mm. The ultrastructure of Alg/Chit/1%Hes gel was analyzed by scanning electron microscope (SEM AIS2100, Seron Technology, South Korea) after sputter coating with gold for 250 s by a sputter coater (SC7620, Quorum Technologies, England) at an accelerating voltage of 20 kV. The average diameters of pores were statistically calculated by using the Image J (National Institutes of Health, Bethesda, USA) and Origin Pro 2015 software (Origin Lab, Northampton, USA) by analyzing a total of 20 random points per image.

2.4.2. Swelling studies

It is well-established that the swelling behavior should be considered when biodegradable materials for drug delivery applications are evaluated [27]. The phosphate-buffered saline (PBS) solution (pH = 7.4), at ambient temperature, was used to evaluate the swelling behavior of Alg/Chit hydrogels, and Equation (1) was used to calculate its amount [28]:

$$\text{Equilibrium mass swelling} = \frac{m_1 - m_0}{m_0} \times 100 \quad (1)$$

where m_0 is the dried mass and m_1 is the swollen mass of the hydrogel. The Alg/Chit hydrogel was lyophilized by using a freeze drier until a constant weight of the gel was reached. Then, a specific amount of hydrogel (4 ml) was immersed in 20 ml of PBS and kept at room temperature for three days. During that period and at the specific time intervals, the specimens were removed from the PBS and quickly weighed. The measurements were performed, and equilibrium mass swelling percentages were calculated from the abovementioned equation.

2.4.3. Weight loss analysis

The degradation rate of the prepared hydrogels was evaluated from their mass loss. Therefore, each dried hydrogel disk was weighed, and equal-weight samples were placed in a falcon tube containing PBS at 37 °C, and the weight loss measurement was performed at 7 and 14 days. At indicated time intervals, triplicate specimens for each group were taken out and dried. Equation (2) was used to determine the degree of degradation in which W_0 is the primary weight of hydrogels and W_1 is the dry weight after removal from water [29].

$$\text{Weight loss } \% = \frac{W0 - W1}{W0} \times 100 \quad (2)$$

2.4.4. Release of hesperidin

The UV–visible spectroscopy was used to evaluate the release of hesperidin from Alg/Chit hydrogel. Alg/Chit/Hes hydrogels (1 mL) were loaded in simulated body fluid (SBF) (5 mL), and the samples were shaken in an incubator shaker under 37 °C at 40 rpm. At various time intervals, the supernatants were extracted and centrifuged, and to determine the amount of released hesperidin, the absorption intensity of supernatants was recorded at 285 nm [30].

2.4.5. Blood compatibility

In this test, 2 ml of human fresh anticoagulated blood diluted with 2.5 ml of normal saline. Next, 0.2 ml of the diluted blood was added to the samples. The mixture worked for 60 min at 37 °C, and then the samples were centrifuged at 1500 rpm for 10 min. The created

supernatants were transferred to a 96-well plate, and the Anthos 2020 (Biochrom, Berlin, Germany) microplate reader was used to measure the absorbance of samples at 545 nm.

In this test, the negative control was 0.2 ml diluted blood in 10 ml normal saline, while the positive control consisted of 0.2 ml diluted blood in 10 ml deionized water. Eventually, hemolysis degree was calculated using Equation (3) [31]:

$$\text{Hemolysis } \% = \frac{Dt - Dnc}{Dpc - Dnc} \times 100 \quad (3)$$

where D_t is the absorbance of the sample, D_{nc} is the absorbance of the negative control, and D_{pc} is the absorbance of the positive control.

2.4.6. Antibacterial assay

The antibacterial properties of the prepared hydrogels against two bacterial species (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) were analyzed by a time-kill assay based on the previous studies [32–34]. Briefly, a bacterial suspension was adjusted to 1×10^7 CFU/ml. The hydrogels were added to the suspension at concentrations of 1/2-fold of the minimum inhibition concentrations (MIC). The suspension (0.5 mL) was incubated at 37 °C with gentle agitation in a shaking water bath. After 24 h of incubation, 10 μL of the suspension was serially-diluted and inoculated on agar plates. After 1, 2, 4, and 24 h of aerobic incubation at 37 °C, the number of viable bacteria colonies was counted. The bacterial growth assays were done in triplicates.

2.5. Cell viability studies

The MTT assay was used to quantitatively assess the viability of cells cultured into the hydrogels. 3T3 murine fibroblast cell line was cultured at the density of 1×10^4 cells on hydrogel in DMEM/F12 supplemented with 10% (v/v) FBS, 100 unit/ml of penicillin and 100 μg/ml of streptomycin in a humidified incubator at 37 °C with 5% CO_2. 1 and 3 days after cells seeding, the culture medium was removed from the 96-well plate and 150 μl of MTT (0.5 mg/ml) was added to each well. Afterward, the cells were incubated at 37° C for 3–4 h in a dark place, and then, the solution was removed, and 0.1 ml DMSO was added to each well. The formed purple formazan crystals were dissolved in DMSO, and the absorption was measured at a wavelength of 570 nm utilizing a microplate reader Anthos 2020 (Biochrom, Berlin, Germany). The positive control was the cells cultured on the tissue culture plate (TCP). All experiments were repeated three times.

2.6. In vivo wound healing study

A full-thickness excisional wound model was used to evaluate the ability of the hydrogels with and without hesperidin in wound healing. Thirty healthy adult male Wistar rats (200–220 g) of 2 months of age were purchased from Pasteur Institute (Tehran, Iran). Animal experiments were approved by the ethics committee of the Iran University of Medical Sciences (ethical code: IR.IUMS.REC.1398.432) and were carried out in accordance with the university's guidelines. Moreover, all animal experiments comply with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). General anesthesia was induced by intraperitoneal injection of the mixture of ketamine (Alfasan, Woerden, The Netherlands; 100 mg/1 kg body weight) and xylazine (Alfasan, Woerden, The Netherlands; 10 mg/1 kg body weight). Afterward, a 1.50×1.50 cm^2 full-thickness wound was excised using a scalpel blade on the back skin of rats near the neck posterior surface. The animals were then randomly divided into five groups (6 rats in each group), and the wounds were treated with alginate/chitosan hydrogel without hesperidin, alginate/chitosan/1% hesperidin, alginate/chitosan/10% hesperidin, and sterile gauze as the negative control. The hydrogels were then embedded on the injury site using an elastic adhesive bandage.

A digital camera (Canon Inc., Tokyo, Japan) at the 7- and 14-days post-surgery was used to evaluate the rate of wound closure by recording the reduction of the wound size. The image analyzing program (Digimizer, Ostend, Belgium) was used to measure the wound area. Finally, the wound closure was calculated via Equation (4) [35]:

$$\text{Wound closure } (\%) = \left(1 - \frac{\textit{Open wound area}}{\textit{Initial wound area}}\right) \times 100 \quad (4)$$

2.6.1. Histopathology study

Six animals from each group were euthanized 14 days post-treatment, and the skin tissues were harvested and immediately fixed in the 10% neutral buffered formalin (PH. 7.26) for 48 h. Then the fixed tissue samples processed, embedded in paraffin, and sectioned to 5 μm thickness. Finally, the sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT). The histological slides were evaluated by the independent reviewer, using light microscopy (Olympus BX51; Olympus, Tokyo, Japan). Epithelialization, angiogenesis, fibroplasia, and granulation tissue formation were assessed in different groups, comparatively.

2.6.2. Histomorphometry analysis

Epithelialization on day 14 was assessed in respect of angiogenesis, and it was scored according to the number of new vessels within the tissue (Figs. 5 and 6), using a 5-point scale as follows: 0 (none), 1 (few), 2 (moderate), 3 (many) or 4. The results of these parameters were reported by a comparative analysis of one independent observer who was blind to the treatment groups.

2.7. Statistical analysis

The Origin Pro software (Version9, OriginLab, Northampton, MA) using a statistic on rows and two-sample *t*-test on rows was used to statistically analyze the results, and the data were expressed as a mean ± standard deviation. In all of the evaluations, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Morphology evaluation

The SEM image was used to microscopically observe the morphology of Alg/Chit/1%Hes hydrogel and evaluate its microstructure (Fig. 1). The SEM image revealed that the prepared hydrogel had a highly porous structure comprised of interconnected pores. The porosity of Alg/Chit/1%Hes hydrogel was calculated as 91.2 ± 5.33%.

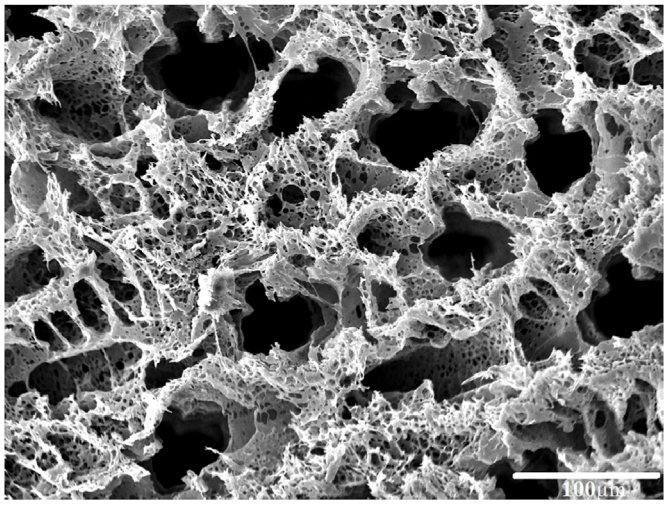

Fig. 1. Scanning electron microscopy of Alg/Chit/1%Hes.

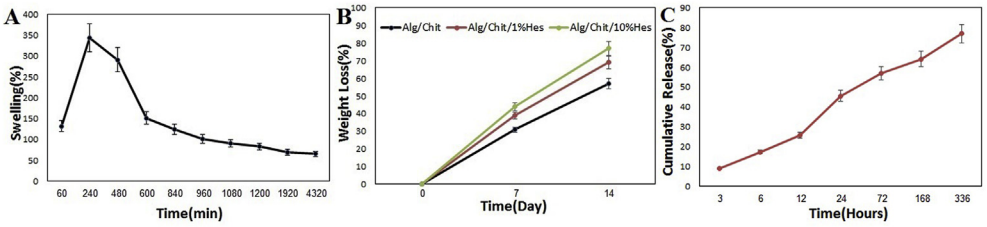

Fig. 2. (A) The swelling percentages of the Alg/Chit hydrogel in PBS at ambient temperature over time, (B) Weight loss percentage of different prepared hydrogels in PBS at 37 °C at different time points (7 and 14 days), (C) Cumulative release profile of hesperidin from the Alg/Chit hydrogel in SBF at 37 °C. Values represent the mean ± SD, n = 3.

The pore size was in the range of 63–144 μm, which is favorable for the cell attachment and migration [36].

3.2. Swelling behavior and weight loss

The swelling behavior of Alg/Chit hydrogel cross-linked with calcium chloride and glutaraldehyde was evaluated from their water uptake value, and the results are shown in Fig. 2A. The interactions between Alg/Chit chains and water molecules could cause the polymer to be swelled while the cross-linking protects the polymer from dissolution. The swelling of Alg/Chit hydrogel provides an appropriate 3D structure that mimics the native microenvironment of cells and supports cell survival, proliferation, and migration. The results show that the highest swelling percentage was 344 ± 12% at 240 min after the incubation. The previous study showed that swelling rate is important to obtain a sustained drug delivery in the wound healing site [37].

The degradation rate of the wound dressing should be matched with the healing rate of the wound. The slow degradation rate hinders wound dresser replacement with the formed tissue. On the other hand, the fast degradation rate might leave the wound site empty, and subsequently, abort the wound healing process. The results of the weight loss for the prepared hydrogels are reported in Fig. 2B. One could infer that the weight loss rate increases to almost 80% upon rising the amount of hesperidin.

3.3. Release

The cumulative release profile of hesperidin is shown in Fig. 2C. It was found that 8.9 ± 1.49% and 17.2 ± 3.67% of the hesperidin has been released within the first 3 and 6 h, respectively, followed by a sustained release of 77.03 ± 8.71% over 14 days.

3.4. Hemocompatibility results

The compatibility of the prepared wound dresser with blood cells, especially erythrocytes, is regarded as a prerequisite for the wound healing [38]. The interaction of the wound dresser with erythrocytes is an initial phase phenomenon after the wound dresser implantation, which determines the following body reactions such as inflammation. The compatibility assay was performed with the interaction between the prepared Alg/Chit with and without hesperidin and fresh erythrocytes which were collected from a healthy volunteer and the released hemoglobin was quantified with a microplate reader. As shown in Fig. 3A, the hemolysis induced by the prepared Alg/Chit hydrogel and Alg/Chit hydrogel with different amounts of hesperidin are significantly lower than positive control, implying the hemocompatibility of the prepared hydrogels.

3.5. Antibacterial properties of the wound dressing

The results of the antibacterial growth assay are reported in Table 1. It was demonstrated that Alg/Chit hydrogel group had a lower number of colonies with regard to the group without wound dressing. By adding hesperidin to the wound dressing, the number of colonies was significantly decreased, and the number of colonies in Alg/Chit/10%Hes group is significantly lower than Alg/Chit/1%Hes group. It was indicated that the Lag growth phase of the two bacteria studied in the Alg/Chit/10%Hes sample was significantly longer than that of the control and Alg/Chit/1%Hes samples.

3.6. Cytotoxicity results

Cytotoxicity of the prepared hydrogels was evaluated using the MTT assay kit at 24 and 72 h after cell seeding, and the results are shown in Fig. 3B. The results reveal that the prepared hydrogels are not only cytocompatible but also have a proliferative effect on the growth of the cells. In Fig. 3B, the positive effect of hesperidin is shown on 3T3 murine fibroblast growth at both incubation times. The cell proliferation on the Alg/Chit/Hes hydrogel is statistically significant compared with TCP at 24 h. It is even significantly higher than Alg/Chit hydrogel 72 h after cell seeding. In addition, by increasing the amount of hesperidin, cell proliferation increases as well. These results suggest that the incorporation of hesperidin into the Alg/Chit hydrogel has made them more suitable for cell proliferation due to its tremendous positive effect on cell growth.

3.7. In vivo wound healing study

Fig. 4 demonstrates the healing effects of the Alg/Chit hydrogel without and with different concentrations of hesperidin as the wound dressings. Fig. 4A shows the macroscopic appearance of wound sites that were covered with a commercial wound dressing (sterile gauze) and Alg/Chit hydrogel without and with different amounts of hesperidin. The image showed that the wound site of Alg/Chit hydrogel group had infection and inflammation, and the healing was incomplete. On the other hand, the groups containing hesperidin treated better rather than the control and Alg/Chit hydrogel groups. Overall, the best performance was observed in the case of Alg/Chit/10%Hes. The image provides that the wound site had no sign of infection and inflammation, and the wound treated very well.

To quantify the wound-healing process wound closure was determined (Fig. 4B). The wound closure of commercial wound dressing was 32 ± 1.66% and 59.1 ± 2.6% after 7- and 14-days post-surgery, respectively. The wound closure of Alg/Chit hydrogel group after 7 and 14 days of surgery was 51 ± 4.38%, 67.8 ± 0.5%, respectively. By adding hesperidin to the prepared hydrogel, the best results were attained for Alg/Chit/10%Hes for which the wound closure was 82 ± 4.2% and 98 ± 1.87% at 7 and 14 days after wounding, respectively.

3.8. Histopathological results

Histologic sections of normal skin tissue were shown in Fig. 5 (A, B, and C). Histopathological evaluation of the negative control group revealed a considerable degenerated neutrophils and eosinophils infiltration. Moreover, the defect site is almost empty, and it covered only by a crusty scab (Fig. 5. E& F-asteroid). Hydrogel without hesperidin showed the initiation of epidermal formation in the defect site. The wound was almost covered with an epidermal layer, and the granulation tissue was evident underneath the new epidermal layer (Fig. 5. I-thick arrow). Histopathology of hydrogel with 1% hesperidin-treated wounds displayed granulation tissue (GT) formation and epidermal

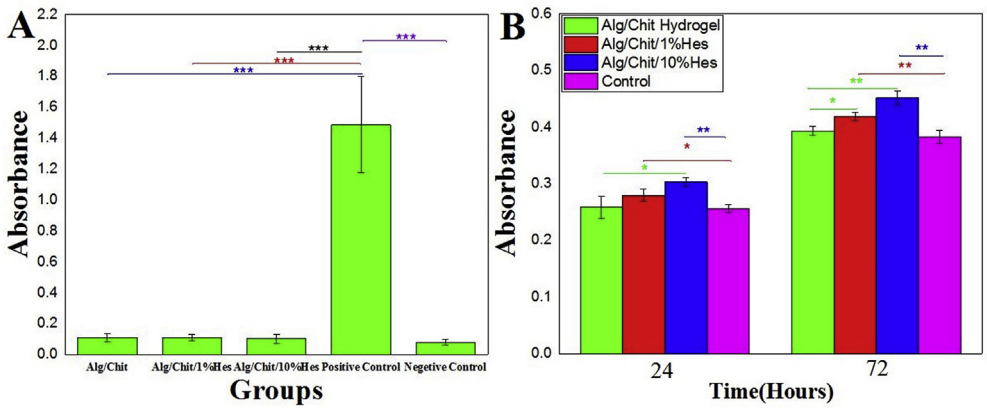

Fig. 3. (A) Blood compatibility histogram of prepared hydrogels, (B) MTT assay histogram after 24 and 72 h post cell seeding, Values represent the mean ± SD, n = 3, *p < .05, **p < .01, and ***p < .001. SD: standard deviation.

proliferation (Fig. 5. L-thick arrow). Hydrogel with 10% hesperidin-tread groups showed wound contraction, epidermal layer formation and remodeling (Fig. 5. O-thick arrow). Although the epidermal layer was completely formed in this group, the hair follicles and appendages (sebaceous gland, etc.) were still absent in this treatment group.

The results of MT staining (Fig. 6) illustrated that among the experimental groups, the hydrogel with 10% hesperidin exhibited a better collagen synthesis and deposition during the wound treatment period. On the other hand, the rate of collagen fiber synthesis and deposition in wounds was the lowest in the case of control negative and hydrogel without hesperidin groups, respectively. It could be inferred that the rate of collagen synthesis was enhanced by increasing the percentage of hesperidin.

3.9. Histomorphometric analysis

The results of histomorphometric analysis after 14 days of skin injury are presented in Table 2. Amongst all groups, re-epithelialization in the negative control group was the minimum, and the group treated with 10% hesperidin showed the highest epidermal proliferation and remodeling ($P < 0.01$). Neovascularization for hesperidin 10% group was significantly higher than other experimental groups ($P < 0.05$). Overall, the healing condition of the hesperidin 10% treated group was better than the hesperidin 1%, Alg/Chit hydrogel, and negative control group on day 14. However, the range of wound healing was moderate in comparison with the positive control group.

4. Discussion

Healing deep wounds becomes a major challenge for wound care specialists. Therefore, specialized therapy is needed to promote proper healing of deep dermal wounded tissues [39,40]. This study aimed to evaluate the combinatorial effects of hydrogel and hesperidin on skin wound healing. To clarify this issue, Alg/Chit hydrogel was prepared and characterized, and then loaded with different amounts of hesperidin, and eventually, the prepared wound dressing was placed in rats after dorsal skin injury. Morphological and functional assessments were used to evaluate the effect of hydrogel and hesperidin on the recovery rate of the injured animals. Different studies have attempted to design and fabricate a practical wound dresser that can not only enhance wound healing but also can accelerate this process [41,42]. Because of their appropriate properties, such as the ability to cool the wound site and having a good structure for cell migration, adhesion, and growth, hydrogels are being widely used in wound healing [43,44]. The composite hydrogels exhibited multiple superiorities which could positively influence the wound healing. Alginate hydrogel has been widely used in wound healing because of its hemostatic ability [45]. Moreover, it has been identified as a substrate for cell proliferation [46,47]. On the other hand, because of its suitable properties like non-toxicity, stability, biodegradability, and biocompatibility, chitosan is a suitable candidate for wound healing applications. Moreover, chitosan hydrogel structures can be loaded with various types of drugs in different physical forms [43]. By using both alginate and chitosan, the hydrogel has excellent gel-forming properties leading to a promising performance in biomedically-relevant hydrogel systems [48].

Hydrogel has a porous structure that can improve cell attachment and migration. Moreover, the interconnected structure could enhance gaseous exchange and provide the passage for nutrients through the diffusion mechanism [49]. The pore size in the range of 20–120 μm has been considered as an appropriate range for the wound healing process [50]. In our study, the average pore size was obtained in the range of 63–144 μm, which was appropriate for cell attachment and migration [36].

Polymers with the ability to form hydrogels have hydrophilic or hydrophobic functional groups. Hydrophilic functional groups provide the ability to absorb water leading to hydrogel expansion; this ability is known as swelling [51]. A previous study indicated that once the swelling process begins, the pore size increases and the structure becomes more suitable for cell attachment and growth [52]. As hesperidin located between Alg/Chit chain and the Alg/Chit hydrogel cannot resist for a long time, it affects swelling, weight loss, and also the release of the prepared hydrogel. The results confirmed this claim and indicated that by increasing the amount of loaded hesperidin, the amount of weight loss increases too.

Table 1
Antimicrobial activity of Alg/Chit hydrogel with different amounts of hesperidin (number of colony forming unit). S.U: *Staphylococcus aureus* PS.a: *Pseudomonas aeruginosa*. Values represent the mean ± SD, n = 3.

Groups	S.U-0h	S.U-1h	S.U-2h	S.U-4h	S.U-24 h	PS.a-0h	PS.a-1h	PS.a-2h	PS.a-4h	PS.a-24 h
Without Wound Dressing	11 ± 0.5	26 ± 1.4	77 ± 1.9	187 ± 3.9	449 ± 5.6	10 ± 1.2	30 ± 2.9	50 ± 2.6	165 ± 3.2	497 ± 9.3
Alg/Chit Hydrogel	12 ± 1.2	26 ± 8.09	73 ± 4.6	159 ± 9.1	415 ± 12.8	11 ± 0.4	23 ± 2.5	29 ± 3.1	109 ± 5.1	355 ± 7.8
Alg/Chit/1%Hes	11 ± 1	19 ± 1.5	58 ± 4.9	125 ± 3.2	401 ± 3.8	12 ± 0.9	20 ± 1.4	27 ± 1.8	77 ± 2.3	299 ± 7.3
Alg/Chit/10%Hes	12 ± 0.8	15 ± 2.7	40 ± 3.3	59 ± 3.7	245 ± 2.7	10 ± 1	15 ± 1.8	19 ± 2.9	50 ± 5.2	128 ± 9.3

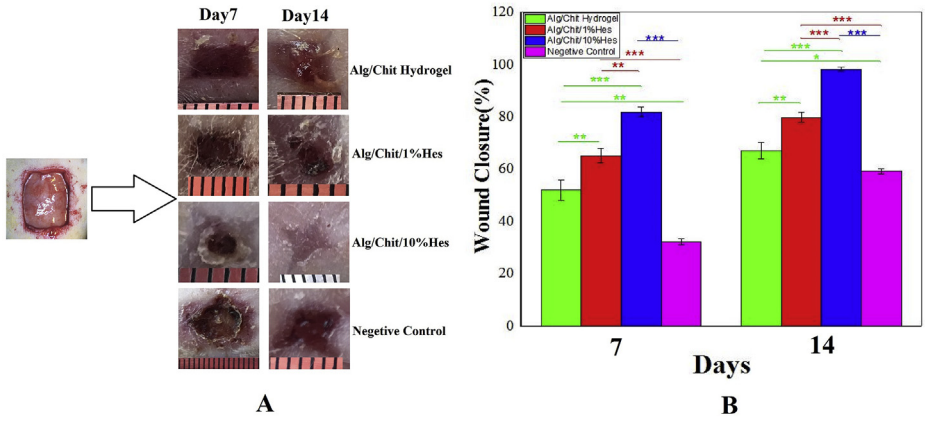

Fig. 4. In vivo wound-healing results: (A) macroscopic appearances of wounds treated 7- and 14-days post-wounding, (B) histogram comparing the wound closure 7th and 14th days post wounding. Values represent the mean ± SD, n = 6, *p < .05, **p < .01, and ***p < .001. SD: standard deviation.

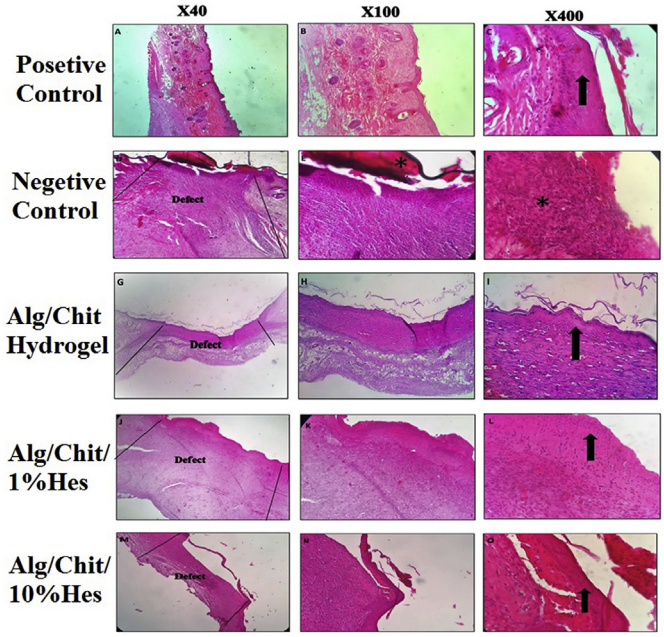

Fig. 5. H&E stained microscopic sections of healed incisions in rats at 14 days.

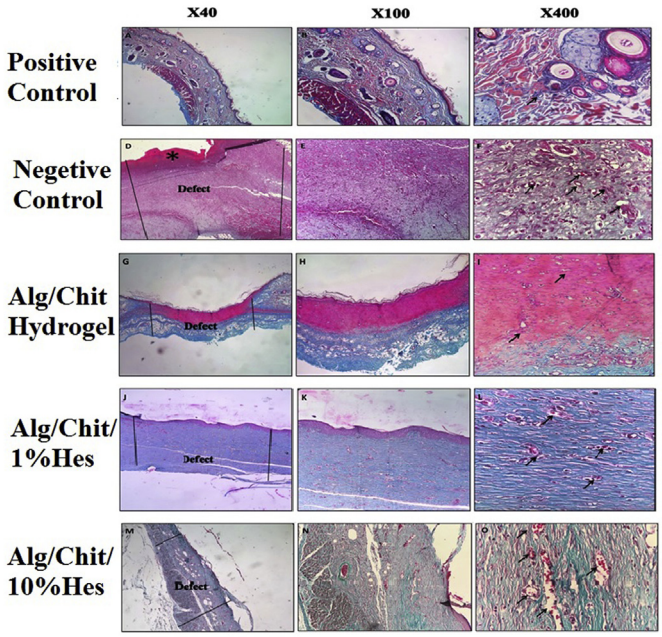

Fig. 6. MT stained microscopic sections of healed incisions in rats at 14 days.

Hemolysis shows the amount of hemoglobin released into the plasma because of damage in erythrocytes. A previous study believes that the hemolysis rate is directly related to the blood compatibility of different materials [39]. In the current study, the hemolysis rate of the Chit/Alg hydrogel with different amounts of hesperidin was the same, and less than the positive control. Therefore, it was found that the hemolysis rate did not change as a result of adding hesperidin to the prepared hydrogel. There is a large body of evidence showing the effect of hesperidin as an anti-inflammatory, antibacterial, antioxidant, anti-radical, diuretic, antiulcer, and antiviral drug implying its decisive role for promoting the healing of excision wounds [19,53–55]. Hesperidin has also been found to improve the strength of capillary, preventing haemorrhages and alleviates infections [56,57]. Experimental data have shown that angiogenesis plays an essential part in the process of wound healing. Haddadi et al. showed hesperidin could stimulate epithelialization, collagen deposition, and cellular proliferation through inducing the VEGF gene, and thus, showed the therapeutic role in improving the wound repair [58].

In this study, the results of the MTT assay showed that hesperidin could increase cell proliferation. Furthermore, the Alg/Chit/10%Hes had the highest cell proliferation between other groups, demonstrating the positive effect of hesperidin. Moreover, the results confirmed that the prepared hydrogel imposed no cytotoxicity effect on the cells.

Table 2
Histomorphometric analysis of different experimental groups. Values represent the mean ± SD, n = 6.

Group	Angiogenesis Score (Mean ± SD)	Epitheliogenesis Score (Mean ± SD)
Negative Control	1.3 ± 0.72	0.23 ± 0.39
Alg/Chit Hydrogel	0.33 ± 0.57	1.40 ± 0.55
Alg/Chit/1% Hes	1.33 ± 0.35	2.66 ± 0.57
Alg/Chit/10% Hes	3.61 ± 1.06	3.45 ± 1.02

In vivo study was used to evaluate the effect of a prepared hydrogel loaded with different amounts of hesperidin on wound healing. The macroscopic evaluation was used, and the results indicated that Alg/Chit/10%Hes had 82 ± 4.2% and 98 ± 1.87% wound healing in 7 and 14 days after surgery which was more than other groups. For further investigation, histopathological and histomorphometric analysis were also used showing that Alg/Chit/10%Hes has better wound

healing than other groups. Several mechanisms have been suggested to explicate the effect of hesperidin on wound healing, namely, scavenging free radicals, suppressing the activation of proinflammatory cytokines such as IL-1β, IL-8, and TNF-α, increasing the capacity of fibroblast, and endothelial cell division which are essential for tissue regeneration of wound [55,59,60]. All in all, the attained results in this study demonstrated the promising potential of Alg/Chit hydrogels loaded with hesperidin for the treatment of skin injuries in clinical conditions.

5. Conclusion

In this study, the effect of Alg/Chit hydrogel with different amounts of hesperidin on the skin wound healing in rat models was evaluated. The results indicated that the prepared hydrogel exhibits appropriate properties for wound healing applications. Besides, in vitro cell growth study confirmed that the cells in the Alg/Chit/Hes groups, especially in the group containing 10% of hesperidin had high cell proliferation rather than the control group. Furthermore, the in vitro evaluation results showed that the Alg/Chit/10%Hes was better than other groups, and almost 95% of the wound was healed after 14 days. The findings of this research suggest that the prepared Alg/Chit/Hes hydrogel-based wound dressings are promising for successful wound treatment.

6. Ethics approval

Animal experiments were approved by the ethics committee of the Iran University of Medical Sciences (ethical code: IR.IUMS.REC.1398.432) and were carried out in accordance with the university's guidelines.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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