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# Preparation and in vivo evaluation of a novel gel-based wound dressing using arginine–alginate surface-modified chitosan nanofibers

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**Abstract**

The development of an effective wound dressing with the ability to induce skin wound healing is a great challenge in medicine. Nanofibers are highly attractive for wound dressing preparation due to their properties such as hemostasis induction, good absorption of wound exudates, and facilitation of cell growth. Chitosan nanofibers have attracted great attention for application in wound dressings due to their accelerating effects on wound healing. In this study, arginine surface-modified chitosan nanofibers were successfully prepared by attachment of arginine molecules on the surface of chitosan nanofibers using sodium alginate through electrostatic interaction. The effect of pH on the amount of attached arginine was evaluated at three different pH values; 5, 6, and 7. Fourier-transform infrared spectroscopy and zeta potential of chitosan nanofibers before and after surface modification suggested the occurrence of the attachment of arginine to chitosan nanofibers. Scanning electron microscope images showed the nanofibrous structure of arginine surface-modified chitosan nanofibers with an average diameter ranging from 100 nm to 150 nm. The release of arginine from arginine surface-modified chitosan nanofibers gel showed a sustained release manner. The suitable viscosity and spreadability of arginine surface-modified chitosan nanofibers gel verified its easy application at the wound site. Arginine surface-modified chitosan nanofibers gel significantly improved the wound healing process including wound closure when tested in vivo using rat model. Additionally, histological examination and immunohistochemical studies showed the significant enhancement of the re-epithelialization, collagen deposition, and angiogenesis in the skin of the animal group treated with arginine surface-modified chitosan nanofibers gel compared with the other control groups. These results suggested that arginine surface-modified chitosan nanofibers gel could be introduced as an effective wound dressing.

**Keywords**

Arginine, chitosan, surface-modified nanofiber, wound healing, alginate

## Introduction

Wound healing is a complex process including different phases: inflammation, cell migration, proliferation, and tissue remodeling.[1] Biological components such as growth factors, cytokines, and nitric oxide (NO) can diffuse through the wound and progress wound healing phases normally.[2,3] Endogenous NO is produced by inducible nitric oxide synthase (iNOS) from arginine at the wound site during wound healing process.[4] NO and its precursors such as arginine can modulate inflammation, stimulate the proliferation of endothelial cells, improve angiogenesis, and enhance collagen deposition and wound contraction.[5,6] Different studies demonstrated that NO-releasing wound dressings can play an important role in wound healing process.[7,8] Kang and coworkers

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prepared a NO-releasing pluronic-based polymer and placed it in a polyethylene glycol (PEG)-based ointment. They used this NO-releasing ointment for the management of acute wounds. The results of their in vivo examinations showed that this ointment could improve reepithelialization, collagen deposition, and blood vessel formation of acute wounds.[7] Blecher et al. reported that NO-loaded silane-based nanoparticle can promote the wound healing by decreasing the inflammation and increasing collagen deposition and blood vessel formation.[8] In addition to using the wound healing agents such as NO, an effective wound dressing can protect the injury, contribute to the recovery of damaged tissues, and improve wound healing process.[9,10] In recent years, nanofiber-based wound dressings have been investigated in wound healing and tissue regeneration studies.[11,12] In comparison with conventional wound dressings, nanofiber-based wound dressings have different advantages such as hemostasis induction, good absorption of wound exudates, and facilitation of cell growth due to their nanofibrous structure.[13–15] Particular properties of nanofibers such as nanometric size, high specific surface area, high drug-loading capacity, and controlled release of the loaded drugs represent them as promising vehicles for drug delivery to wounds.[16]

Chitosan is a nontoxic, antibacterial, biodegradable, and biocompatible biopolymer. Due to its properties, chitosan is widely used for biomedical applications such as drug delivery, tissue engineering scaffolds, and wound healing dressings.[17] Chitosan nanofibers can be produced by electrospinning method or by mechanical and chemical treatment.[18] The use of chitosan nanofibers in the various fields such as tissue engineering and wound healing had been reported.[17,19] Dispersion of chitosan nanofibers in water can form a viscous gel that is suitable for topical applications. Chitosan nanofibers gel can form at low concentration (about 2.5% w/w) by the hydration of chitosan nanofibers in water. There are high amount of amino groups on the surface of chitosan nanofibers that can be used for the formation of hydrogen or electrostatic bonds with different drugs and polymers.[20] The formation of electrostatic bonds between drugs and nanofibers can sustain the release of attached drugs. Moreover, nanofibers can form matrix and thus control and prolong the release of drugs that are attached on the nanofibers surface.[21] Controlled delivery of wound healing agents from wound dressing can improve patient compliance and therapeutic outcomes.[22] In addition, chitosan nanofiber gel can be sterilized easily by autoclave. These properties indicate that the chitosan nanofibers gel can be used as a promising material for preparation of advanced wound dressings. Advanced wound dressings contain wound healing agents and show biological activity because of bioactive constituents or drugs that are incorporated within these dressings.[15] Following this rationale, in current study, we prepared arginine surface-modified chitosan nanofibers gel as a promising wound dressing with easy application for the treatment of acute wounds. Arginine (as a NO precursor) was attached on the surface of chitosan nanofibers by electrostatic interactions using sodium alginate polymer. Structural and physicochemical properties of arginine surface-modified chitosan nanofibers gel were investigated, and its wound healing properties as wound healing dressing were evaluated in vivo.

## Material and methods

### Materials

The chitosan nanofibers aqueous gel (2.5% w/w) was purchased from Nano Novin Polymer Co (Mazandaran, Iran). Arginine and sodium alginate (molecular weight: 10,000 Da) were purchased from Merck chemical company (Germany). Acetic acid was obtained from the BASF Company (Germany). Deionized water was used throughout the experiment. All other chemicals used were of reagent grade.

### Preparation of chitosan nanofibers aqueous gel (Chi-NFs gel; 2.5% w/w)

The Chi-NFs gel (2.5% w/w) that was purchased from Nano Novin Polymer Co was prepared by chemical and mechanical method.[23] The deacetylation degree of chitosan was reported between 80% and 85%.

### Preparation of Chi-NFs gel at different pH values

The pH of Chi-NFs gel (2.5% w/w) was determined using pH meter. The pH of Chi-NFs gel (2.5% w/w) was recorded at $7 \pm 0.1$. Then few drops of acetic acid 10% were added to 1 g of Chi-NFs gel (2.5% w/w) to obtain Chi-NFs gel with pH 5 and 6.

### Determination of zeta potential of Chi-NFs gels at different pH values

The zeta potential of Chi-NFs gels at different pH values was measured using by Zetasizer (Zetasizer 3600, Malvern Instrument Ltd, Worcestershire, UK) at 25°C. The concentrations of all measured samples remain the same at 0.05 wt%.[24]

### Preparation of arginine surface-modified Chi-NFs gels (Arg-Chi-NFs gels) at different pH values

Arginine was dissolved in distilled water to obtain a solution with the concentration of 200 mg/mL. Three milliliters of prepared arginine solution (200 mg/mL)

**Table 1.** The amounts of attached arginine on the surface of Chi-NFs and the zeta potential of Arg-Chi-NFs at different pHs ($n = 3 \pm$ SD).

| Sample | F1 | F2 | F3 | F4 | F5 | F6 |
|---|---|---|---|---|---|---|
| pH | 5 | 5 | 6 | 6 | 7 | 7 |
| Amount of arginine added to 25 mg of Chi-NFs (mg) | – | 200 | – | 200 | – | 200 |
| Amount of sodium alginate added to 25 mg of Chi-NFs (mg) | – | 100 | – | 100 | – | 100 |
| Amount of attached arginine to 25 mg of Chi-NFs (mg) | – | $180.32 \pm 2.65$ | – | $194.28 \pm 2.01$ | – | $191.88 \pm 2.38$ |
| Zeta potential | $+40.21 \pm 0.44$ | $+39.5 \pm 0.21$ | $+33.15 \pm 0.14$ | $+31.8 \pm 0.09$ | $+20.75 \pm 0.31$ | $+19.00 \pm 0.2$ |
| Increase in zeta potential | – | $0.71 \pm 0.23$ | – | $1.35 \pm 0.07$ | – | $1.75 \pm 0.11$ |

were added to 3 mL of sodium alginate solution (10% w/v in deionized water) and were stirred for 4 h until arginine molecules were conjugated to sodium alginate by electrostatic interaction. Then 2 mL of prepared arginine–sodium alginate complex solution was added to amount of Chi-NFs gel (at different pH 5, 6, and 7) that contain 25 mg of Chi-NFs. The pH adjustment of Chi-NFs gels to 5, 6, and 7 (if necessary) was also performed after addition of arginine–sodium alginate complex solution to Chi-NFs gels. The prepared mixtures of arginine–sodium alginate complex solution and Chi-NFs gel at different pH (5, 6, and 7) were incubated at room temperature using a laboratory magnet stirrer at a shaking frequency of 50 r/min for 24 h until the arginine–sodium alginate complexes were attached to Chi-NFs by electrostatic interaction. Then, these mixtures were centrifuged (Sigma Laboratory Centrifuge, Model 3 K-30; Sigma Laborzentrifugen GmbH, Osterode, Germany) to sediment the Arg-Chi-NFs gels. For separation of unbound arginine, 2 mL of deionized water was added to sediment and mixed carefully. Then the prepared mixture was centrifuged to sediment the rinsed Arg-Chi-NFs gel. The resulting supernatants were filtered through a 0.22-μm syringe filter (Merck Millipore, Darmstadt, Germany). The concentration of unbound fraction of arginine was determined from the filtered supernatants spectrophotometrically (UV spectrophotometer, UV mini 1240, Shimadzu, Kyoto, Japan). The amount of arginine bound to 25 mg of Chi-NFs at different pH values was then calculated as the difference in the amount of the arginine added to the Chi-NFs gel and the amount of unbound arginine and presented as average value of three repetitions (Table 1). Then the Arg-Chi-NFs gels were collected after centrifuge and their weights were determined precisely. If necessary, sufficient amount of deionized water (pH 7) or acetic acid solution (pH 5 or 6) was added to Arg-Chi-NFs gels until their weights reach to 1 g.

### *Fourier transform infrared spectroscopy*

Chi-NFs gel and Arg-Chi-NFs gel were dried using freeze dryer (Christ alpha 2–4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The operating conditions were at the temperature of $-40^{\circ}$C and pressure of 0.001 bar. Lyophilization time lasted for 48 h. Pure arginine, sodium alginate powder, dried Chi-NFs gel, and dried Arg-Chi-NFs gel were diluted with potassium bromide and made into pellets. The FTIR spectra of samples were recorded using a FTIR spectrometer (FT/IR-6300, JASCO, Tokyo, Japan) at wave numbers 400–4000 $cm^{-1}$ to confirm the electrostatic attachment of arginine on the surface of Chi-NFs.

### *Determination of the release of arginine from Arg-Chi-NFs gel in 0.2 M phosphate buffer at pH 7.4*

The release of arginine from Arg-Chi-NFs gel was measured in 0.2 M phosphate buffer at pH 7.4. To do this, 1 g of Arg-Chi-NFs gel was placed in dialysis tube (cellulose membrane, cutoff 12,000 Da, Merck, Germany) and the dialysis tube was placed into wide mouth jar containing 25 mL of phosphate buffer at pH 7.4 as release medium. The temperature and stirring rate of the system were maintained at 32°C and 100 r/min, respectively.[25]At the predetermined time intervals, an aliquot sample of release medium was withdrawn and the same volume of fresh medium was added back to the release medium. The concentration of arginine was quantified spectrophotometrically (UV mini 1240, Shimadzu, Kyoto, Japan).

### *Viscosity measurement and spreadability test of Chi-NFs gel and Arg-Chi-NFs gel*

The viscosity of the Chi-NFs gel was measured using a Brookfield DV-III ultraprogrammable rheometer

(Brookfield Engineering Laboratories, Middleboro, MA; spindle 74, 50 r/min) at room temperature before and after surface modification with arginine. To determine the spreadability of formulations, 0.5 g of Chi-NFs gel or Arg-Chi-NFs gel was placed within a circle of 1 cm diameter pre-marked on a glass plate of $20 \times 20$ cm, over which a second glass plate was placed. A weight of 500 g was allowed to rest on the upper glass plate for 5 min. Spreadability was determined as a difference in diameter values before and after 30 s on a centimeter scale.[26]

### *Field scanning electron microscopy (FESEM)*

The morphologies of Chi-NFs and Arg-Chi-NFs were studied using a field emission scanning electron microscopy (FESEM HITACHI S-4160, HITACHI, Tokyo, Japan) at an acceleration voltage of 20 kV. The Chi-NFs gel and Arg-Chi-NFs gel were dried at room temperature and coated with gold to prevent charging.

### *In vivo wound healing assay*

All animal experiments were done in accordance with protocols approved by the ethics committee of the Pharmaceutical Research Centre, Faculty of Pharmacy, Isfahan University of Medical Sciences, Iran. Wistar rats ($250 \pm 25$ g, male, Pasteur Institute, Tehran, Iran) were housed under standard conditions of controlled temperature and humidity ($25^{\circ}C \pm 1$, 40%) and 12:12-h dark/light cycle. The experiments were performed during the light phase (from 09.00 a.m. to 11.00 a.m.) of the cycle. During the study, animals had unlimited access to food and water. Before the wounding, the dorsal hairs of rats were removed with an electric shaver under ketamine/xylazine anesthesia. A full thickness skin wound of 1.5 $cm^2$ was prepared by excising the dorsum of rats using surgical scissors and forceps (day 0). The animals were divided into four groups. There were six animals in each group. The animals were housed individually under standardized environmental conditions. In the test group 1, wounds were treated with about 1 g of Arg-Chi-NFs gel (F6) that contains 200 mg of arginine. In the test group 2, wounds were treated with about 1 g of the physical mixture of Chi-NFs gel and sodium alginate (the amount of sodium alginate and Chi-NFs was equal to the amount of these materials in the samples of group 1). In test group 3, wounds were treated with 1 mL of arginine solution (200 mg/mL). In test group 4, wounds were treated with 1 mL of normal saline solution. Each animal received treatment once a day in the experimental period (9 days). On days 0, 4, and 9 of study, the wounds were photographed by a digital camera and the size of wounds was determined using Adobe Photoshop® software, and compared relative to the wound size of the first day. The wound closure was calculated using bellow equation.

Wound closure (%) = ((Area on day 0 − Area on day n)/Area on day 0) × 100 (%)

On days 4 and 9, three rats of each group were sacrificed and the skin wound tissues were carried out following surgery using sterile surgical tools for histological evaluation.[7]

### *Histological examination and immunohistochemical studies*

The skin wound tissue of individual rats was excised and fixed in a 10% formalin solution. The wound tissues were then dehydrated with graded ethanol and xylene and then embedded in paraffin block to prepare tissue sections. Five micrometer sections have been cut from wound tissue blocks using a manual microtome. The sections were stained by hematoxylin and eosin (H&E) for morphological analysis and by Masson's Trichrome for collagen deposition evaluation. Stained sections were examined and imaged using a light microscope and camera (Nikon). The reepithelialization process was analyzed using the scale established in previous studies.[27] The scores were 0 for reepithelization at the margin of the wound, 1 for reepithelization covering less than half of the wound, 2 for reepithelization covering more than half of the wound, 3 for reepithelization covering the entire wound with irregular thickness, and 4 for reepithelization covering the entire wound with normal thickness. The deposition of collagen was determined following the scale established in previous studies.[28] Zero, absence of collagen; 1, mild content of collagen; 2, moderate content of collagen and 3, marked content of collagen. In order to evaluate neoangiogenesis in the wound bed, immunohistochemical studies were performed. The number of vessels in the wound bed was quantified using anti-CD31 monoclonal antibody (Roche, USA).[29] Analysis of stained sections was performed by an experienced pathologist.

## Statistical analysis

All statistical analysis was performed using Statistical Package for the Social Sciences version 16 software (SPSS Inc, Chicago, IL, USA). The statistical significance of differences was determined by one-way analysis of variance. All statistical tests were two-sided, and $P$ values less than 0.05 were considered to be statistically significant. The results are shown as mean ± standard deviation.

## Results and discussion

### *Characterization of Arg-Chi-NFs gels at different pH values*

Arginine is a semi-essential amino acid in humans, which can be converted via nitric oxide synthase (NOS) to NO. Arginine is a basic amino acid that has a positive charge at pH ranges from 5 to 7 (L-arginine isoelectric point (pI): 10.8).[30] Chitosan is a natural biocompatible polymer that is prepared by deacetylation of chitin and has free amino groups along its chains. According to Sorlier et al. study, a higher degree of deacetylation would lead to the preparation of chitosan with larger pKa. It was recorded that for varying degree of deacetylation from 5% to 75%, pKa of chitosan varies between 6.3 and 7.2. Whereas the degree of deacetylation of chitosan used for preparation of Chi-NFs was above 80%, thus the pKa of used Chi-NFs is above 7.2.[31] Chitosan shows positive charge at a pH less than its pKa due to the protonation of its amino groups. Thereby all Chi-NFs in prepared gels with different pH values (5, 6, and 7) have positive charge. Whereas, both arginine and Chi-NFs have positive charge over pH range from 5 to 7, thus arginine cannot attach on the surface of Chi-NFs using electrostatic interactions. For attachment of arginine on the surface of Chi-NFs, we should use a water-soluble and negative-charged polymer such as sodium alginate. Sodium alginate is used for preparation of alginate wound dressings extensively.[32] The pKa value of sodium alginate is in the range of 3.4–4.4. Thereby at pH 4.5, due to presence of ionizable carboxylic acid groups along the sodium alginate chain, this polymer has strongly negative charge. It is expected that the negative charge of sodium alginate increased at higher pH.[33] During the preparation Arg-Chi-NFs gels, firstly arginine molecules were attached to sodium alginate by electrostatic interaction. The prepared arginine–sodium alginate complex showed a negative zeta potential at different pH and thereby a negative charge. The zeta potential of arginine-sodium alginate complex was $-5.82 \pm 0.25$, $-4.35 \pm 0.75$, and $-3.24 \pm 0.67$ at pH 7, 6, and 5 respectively. In addition, the zeta potential of the Chi-NFs was $+41.21 \pm 2.44$, $+31.15 \pm 1.17$, and $+20.75 \pm 3.26$ at pH 5, 6, and 7, respectively. In the lower pH values, more number of amino groups of Chi-NFs will be ionized; consequently, the positive charge of Chi-NFs will be increased.[34] Similar values have been reported for Chi-NFs in other studies.[35] Thus, negative-charged arginine–sodium alginate complex can be attached to the positive-charged Chi-NFs by electrostatic interaction. The preliminary confirmation of electrostatic interaction between arginine–sodium alginate complex and Chi-NFs can be discerned easily by the shift of the zeta potential of the Chi-NFs after the surface modification with arginine–sodium alginate complex. Zeta potential of Chi-NFs at different pH decreased after the surface modification with arginine–sodium alginate (Table 1). The decrease in the zeta potential of Chi-NFs indicates that some of positive-charged groups of Chi-NFs had been neutralized after electrostatic interaction with negatively charged groups of arginine–sodium alginate complex. The maximum decrease in the zeta potential value ($1.75 \pm 0.11$) was recorded in case of addition of arginine–sodium alginate complex to 25 mg of Chi-NFs at pH of 7 (F6). It may be related to higher negative charge of sodium alginate at pH 7. The amount of attached arginine on the surface of Chi-NFs at different pH values was recorded in Table 1. As seen in Table 1, the maximum amount of attached arginine on the surface of Chi-NFs ($194.28 \pm 2.01$ mg) was recorded in the case of addition of 200 mg arginine to 25 mg of Chi-NFs at pH of 6 (F4). Thus, the optimum pH for electrostatic interaction between l-arginine and sodium alginate or Chi-NFs and l-arginine-sodium alginate mixture is 6. It can be attributed that the difference between pH and pka of Chi-NFs and sodium alginate is approximately equal and arginine molecules have sufficient positive charge for good attachment to sodium alginate chain and ultimately to Chi-NFs. Thus in the best situation, there is $194.28 \pm 2.01$ mg arginine in 1 g of Arg-Chi-NFs gel at pH 6 (F4). The high amount of attached arginine on the surface of Chi-NFs can be related to high aspect ratio of Chi-NFs. Although the maximum attached arginine was achieved for sample F4 (pH 6), but there is no significant difference between amount of attached arginine in F4 and amount of attached arginine in F6 (pH 7; *P* value ? 0.05). There is $191.88 \pm 2.38$ mg arginine in 1 g of Arg-Chi-NFs gel at pH 6 (F4). In this study, we did not prepare Arg-Chi-NFs gels with more than 200 mg of L-arginine in 1 g of Arg-Chi-NFs gel. According to Matsumoto and Kuroyanagi study,[36] the arginine content more than 200 mg in each topical dose has a negative effect on reepithelization and cause intense inflammation. This suggests that the amount of arginine in prepared Arg-Chi-NFs gels is suitable. In our study, F6 is more preferable compared with the other formulations (F2 and F4). Because there is sufficient amount of attached arginine in each gram of F6 and in addition, the pH of Chi-NFs gel that used for preparation of F6 is 7, thus no pH changes are required in the preparation process of this sample. Thus, the preparation process of F6 is easier than other formulations. Thus, F6 was selected as the optimum formulation for rest of our study.

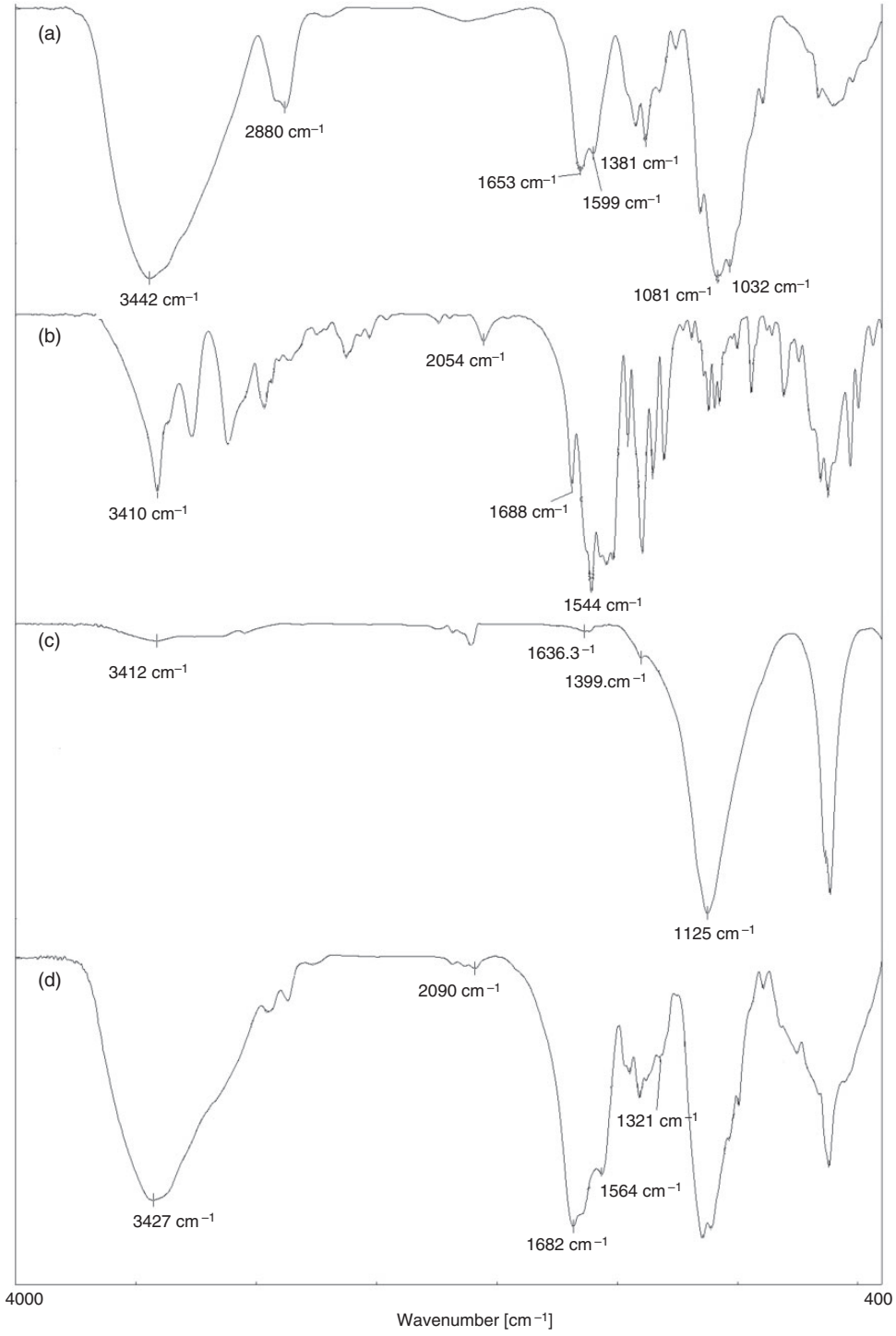


**Figure 1.** FTIR spectra of the Chi-NFs (a), arginine (b), sodium alginate (c), and Arg-Chi-NFs (F6) (d).

## *Fourier transform infrared spectroscopy*

Figure 1 depicts the FTIR spectra of the Chi-NFs (a), arginine (b), sodium alginate (c), and Arg-Chi-NFs (F6) (d). In the spectra of Chi-NFs, the characteristic peaks at 1653 and 1599 $cm^{-1}$ corresponded to C = O stretching of the secondary amide and NH2 bending of the primary amine groups, respectively. The Chi-NFs sample presented two peaks around 1032 and 1081 $cm^{-1}$attributed to primary and secondary amino group vibration bends, respectively. Other principal absorption peaks appeared at 3442, 2880,

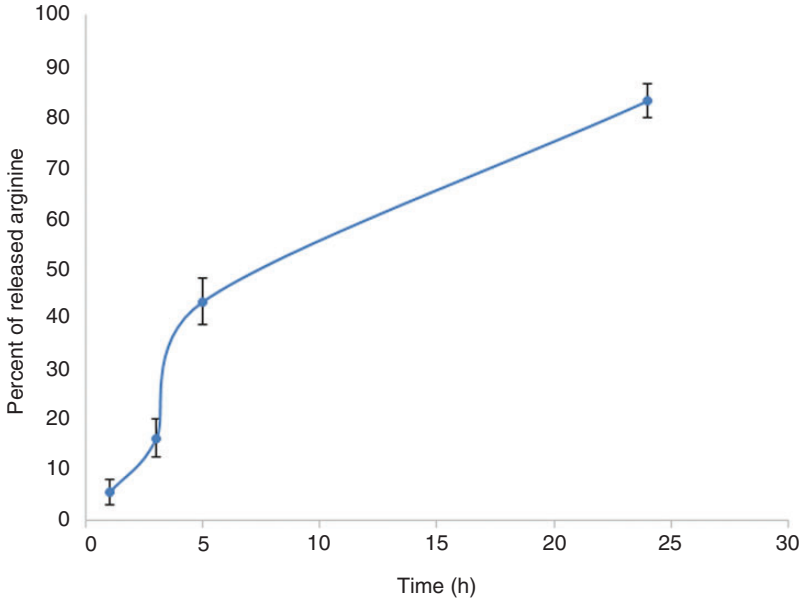


**Figure 2.** The release profile of arginine from Arg-Chi-NFs gel (F6) in phosphate buffer pH 7.4. The vertical bars indicate the standard deviations ($n = 3$).

and 1381 $cm^{-1}$ due to the OH stretching vibration, $CH_2$ asymmetrical stretching vibration, and vibration bends (Figure 1(a)).[37] In the FTIR spectrum of arginine (Figure 1(b)), the two principal absorption peaks were appeared at 1544 and 1688 $cm^{-1}$ due to the N–H bending vibration of imine group and C = O stretching of carboxylic groups. The absorption bands between 2100 and 3500 $cm^{-1}$ are due to overlapping of peaks of the stretching N–H vibration of primary amine, CH stretching, and OH stretching of carboxylic acid group.[38] In the FTIR spectrum of sodium alginate (Figure 1(c)), the two peaks appeared at 1636 and 1399 $cm^{-1}$ due to asymmetric and symmetric stretching vibrations of carboxylate salt ion, respectively. The stretching O–H bonds appeared in the range of 3000–3600 $cm^{-1}$. The band at 1125 $cm^{-1}$ was attributed to the C–O stretching vibration of pyranosyl ring and attributed to its saccharide structure.[39] Figure 1(d) shows the FTIR spectrum of Arg-Chi-NFs. There are some changes in the absorption bands of the FTIR spectra in the Arg-Chi-NFs compared to Chi-NFs, sodium alginate, and arginine spectra. Absorption bands at 1599 $cm^{-1}$ (NH2 bending of the primary amine group) for Chi-NFs and 1399 $cm^{-1}$ (symmetric stretching vibrations of carboxylate salt ion) for sodium alginate may be used to monitor the complex formation between Chi-NFs and sodium alginate because they are characteristic peaks of these polymers. The FT-IR spectrum of the Arg-Chi-NFs showed that the band at 1599 $cm^{-1}$ for Chi-NFs shifted to 1564 $cm^{-1}$ and the band at 1399.1 $cm^{-1}$ for sodium alginate shifted to 1321.96 $cm^{-1}$, which indicates the electrostatic interaction between Chi-NFs and sodium alginate. Absorption band at 1544 $cm^{-1}$ (N–H bending vibration of imine group) for arginine was overlapped with absorption band of primary amine groups of the Chi-NFs. This overlap hinders the characteristic peak of arginine. But other characteristic bands of arginine remained approximately unchanged (1682 $cm^{-1}$due to C = O stretching of carboxylic groups and broad spectrum between 2100 and 3500 $cm^{-1}$ for N–H, C–H, and O–H stretching absorption), which clearly indicates the presence of arginine in the composition of Arg-Chi-NFs.[40,41]

## *The release of arginine from Arg-Chi-NFs gel in 0.2 M phosphate buffer at pH 7.4*

To determine the ability of Chi-NFs to control arginine release, the release behavior of arginine from the Arg-Chi-NFs (F6) at pH 7.4 was studied. Figure 2 shows the release profile of arginine from Arg-Chi-NFs (F6) in phosphate buffer pH 7.4. As shown, 6.99 ± 2.51% of arginine was released within the first hour, followed by a slow and sustained release manner. Approximately, 85.35 ± 3.40% of bound arginine released after 24 h at pH 7.4. The slow release of arginine from the Arg-Chi-NFs is due to the electrostatic interaction between arginine and sodium alginate and arginine–alginate complex with Chi-NFs.[42] The slow release of wound healing agents such as NO from wound dressing can improve the wound healing rate significantly in comparison with the fast release patterns of wound healing agents.[22,43]

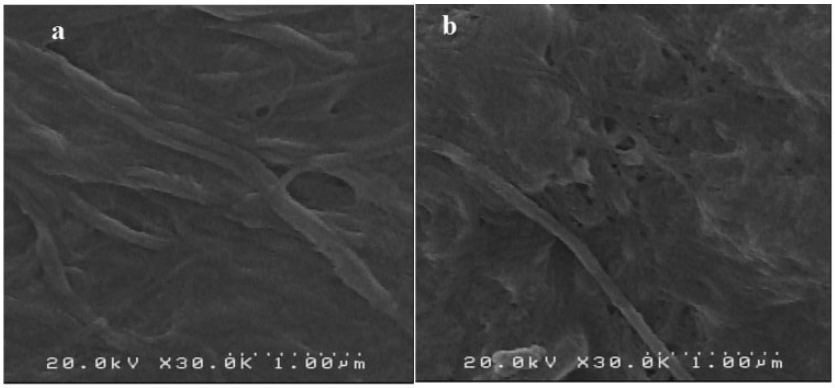


**Figure 3.** FESEM images of Chi-NFs (a) and Arg-Chi-NFs (b).

### *Viscosity measurement and spreadability test of Arg-Chi-NFs gel*

Viscosity measurement and spreadability test were performed to obtain reliable information about the ease of using the prepared wound dressing. Dispersion of Chi-NFs and Arg-Chi-NFs in water can form a viscous gel. These gels can form at low concentrations of these nanofibers (2.5% w/w) by dispersed and hydrated entangled fibrils. The viscosity of Chi-NFs gel (2.5 w/w%) at pH 7 (F5) and Arg-Chi-NFs gel (2.5 w/w%) (F6) was $1007 \pm 12$ cps and $1000 \pm 10$ cps, respectively. As seen the viscosity of Cel-NFs gel (2.5 w/w%) did not change significantly after surface modification with arginine ($P$ value ? 0.05). The viscosities of the Chi-NFs gel (2.5 w/w%) and Arg-Chi-NFs (2.5 w/w%) (F6) were similar to the other prepared topical gels exhibit more suitability of Chi-NFs gel (2.5 w/w%) and Arg-Chi-NFs (2.5 w/w%) (F6) for topical application.[44,45] The high viscosity of hydrocolloid wound dressings can help to wound dressing to remain in the wound cavity.[46] Spreadability is one of the important characteristics of a topical formulation that should be evaluated especially in the formulations with high viscosity. The suitable spreading is important for both clinician (more therapeutic efficacy of the formulation) and patient (more comfortable and greater compliance and lower pain). The extent of area which the topical formulation easily cover the affected skin can reflect the ease of spreading. The spreadability of the Chi-NFs gel (2.5 w/w%) and Arg-Chi-NFs (2.5 w/w%) (F6) was $7.1 \pm 0.3$ and $8.3 \pm 0.5$ cm respectively, which proved the ease of applicability of gels on skin.[46] The higher spreadability of the Arg-Chi-NFs (2.5 w/w%) (F6) compared to Chi-NFs gel (2.5 w/w%) may be related to its lower viscosity.

### *Field scanning electron microscopy (FESEM)*

FESEM images of Chi-NFs and Arg-Chi-NFs are shown in Figure 3(a) and (b), respectively. The FESEM images showed that both Chi-NFs and Arg-Chi-NFs had fibrous structure. The Chi-NFs had diameters ranging from 80 to 120 nm. The Arg-Chi-NFs had diameters ranging from 100 to 150 nm. Chi-NFs had more uniform structure than Arg-Chi-NFs. The results show that the average Chi-NFs diameter increased slightly after surface modification with arginine–sodium alginate complex.

### *In vivo wound healing assay*

Wound contraction occurs between 7 and 14 days after injury. Faster wound closure can reduce pain, prevent infection, and scar formation.[47] Thus, the wound healing efficacy of the Arg-Chi-NFs gel (F6), Chi-NFs gel (F5)–sodium alginate mixture, arginine solution, and normal saline solution was evaluated by calculating the percent wound closure through the measurement of the wound area ($mm^2$) on the day of surgery and on days 4 and 9 after surgery (Figure 4). On day 4, significant differences were found among the Arg-Chi-NFs gel treated group and other groups. As shown in Figure 4, after nine days; Arg-Chi-NFs gel (F6) significantly accelerated the closure of full-thickness excisional wounds compared to other groups. The wound area of group treated with Arg-Chi-NFs gel (F6) was decreased by $93.8 \pm 3.1\%$ after nine days relative to day 0, compared to $76.5 \pm 4.5\%$ in group treated with the Chi-NFs gel (F5)–sodium alginate mixture, $60.4 \pm 2.5\%$ in group treated with arginine solution and $51.2 \pm 3.9\%$ in normal saline-treated group. Moreover, group that treated with Chi-NFs gel (F5)–alginate mixture showed suitable increase of wound closure possibly due to wound healing effects of chitosan and sodium alginate and because Chi-NFs gel–alginate mixture can provide a moist environment around wound.[48] Arginine solution increased wound closure rate compared to normal saline because of its wound healing effect.[6,7] These results are in agreement

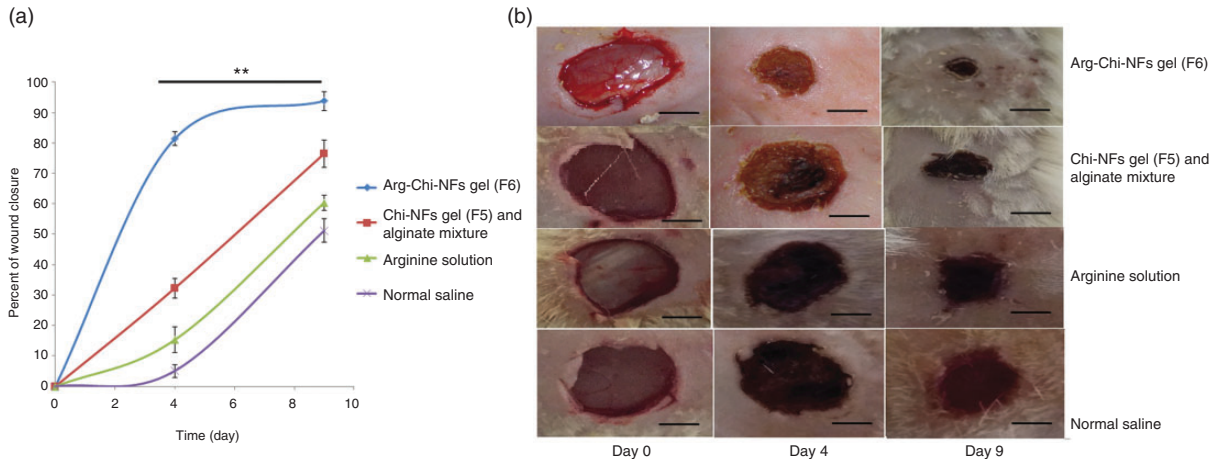


**Figure 4.** (a) Effect of Arg-Chi-NFs gel, Chi-NFs gel, arginine solution, and normal saline on wound closure. Values are presented as a percentage of the wound area compared to day 0 (mean ± SEM). **Significantly greater than other groups ($P < 0.05$); (b) photographs of macroscopic appearance of wounds treated with Arg-Chi-NFs gel (F6), Chi-NFs gel (F5), arginine solution and normal saline; at the surgery day and 4 and 9 days after surgery day, scale bar, 500 μm.

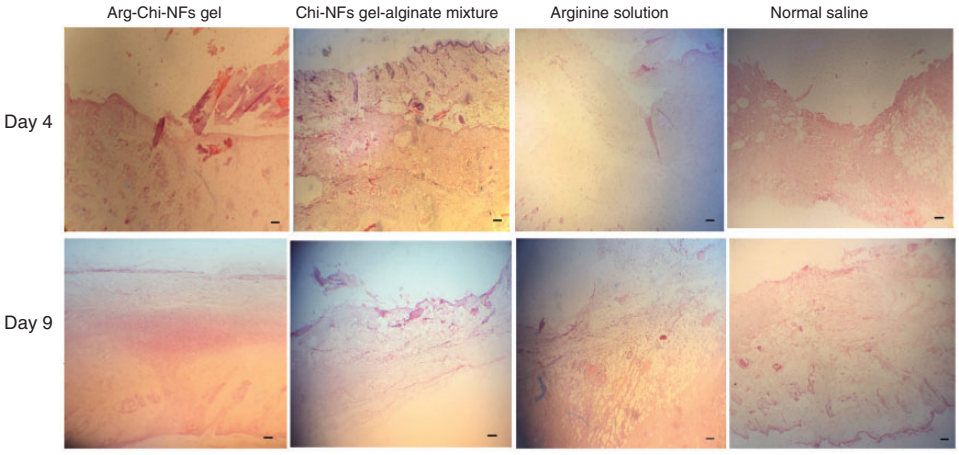


**Figure 5.** Representative histological images of skin wounds treated with Arg-Chi-NFs gel (F6), Chi-NFs gel (F5)–sodium alginate mixture, arginine solution, and normal saline at 4 and 9 days after surgery day stained with hematoxylin and eosin (H&E), magnification: 40×, scale bar, 75 μm.

with the wound area reduction observed in the digital images of the wounds of each group from days 0, 4, and 9 shown in Figure 4. A significant improvement in wound closure was obtained after using Arg-Chi-NFs gel (F6) compared to other groups from day 4 to day 9. These results suggest that Arg-Chi-NFs gel can improve wound closure and healing by releasing NO at the wound site especially during the early proliferative healing.[49]

## *Histological examination and immunohistochemical studies*

*Reepithelialization.* In addition to wound closure, the treatment effectiveness of Arg-Chi-NFs gel was also evaluated through histological analysis. The reepithelization or covering of the wound surface with the new epithelium is the main process of wound healing in humans.[49] To confirm the reepithelialization, wound tissue sections were stained with H&E (Figure 5). On day 4, wound scabs were seen on the wound tissue of Arg-Chi-NFs gel group that protect wounds from attacking any pathogens outside.[1,49] From the H&E stains of day 4, it was noted that Arg-Chi-NFs gel (F6)-treated group had an earlier onset of reepithelization compared to other groups. Moreover, the results showed that Arg-Chi-NFs gel (F6)-treated group presented a significantly higher grade of reepithelization after nine days. The score of the reepithelization in Arg-Chi-NFs gel (F6)-treated group according to the scores described by Sinha and Gallagher[27] was around 3 and 4 after four and nine days of surgery and

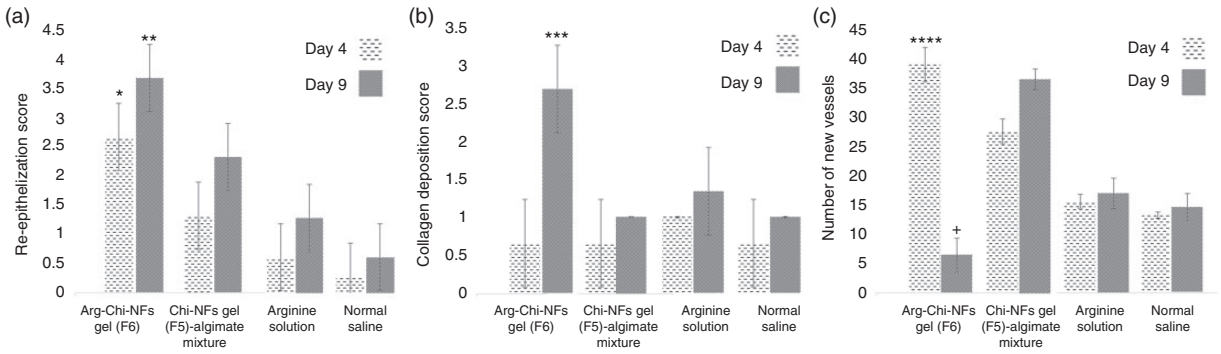


**Figure 6.** Histological analysis. (a) Reepithelization score, *significantly greater than other groups at day 4 after surgery ($P$ value $< 0.05$), **significantly greater than other groups at day 9 after surgery ($P$ value $< 0.05$), (b) score of collagen deposition, ***significantly greater than other groups at day 9 after surgery ($P$ value $< 0.05$) (c) number of new vessels in immunohistochemically stained tissue slides, ****significantly greater than other groups at day 4 after surgery ($P$ value $< 0.05$); + significantly lower than other groups at day 9 after surgery ($P$ value $< 0.05$). All the results are shown as mean $\pm$ SD.

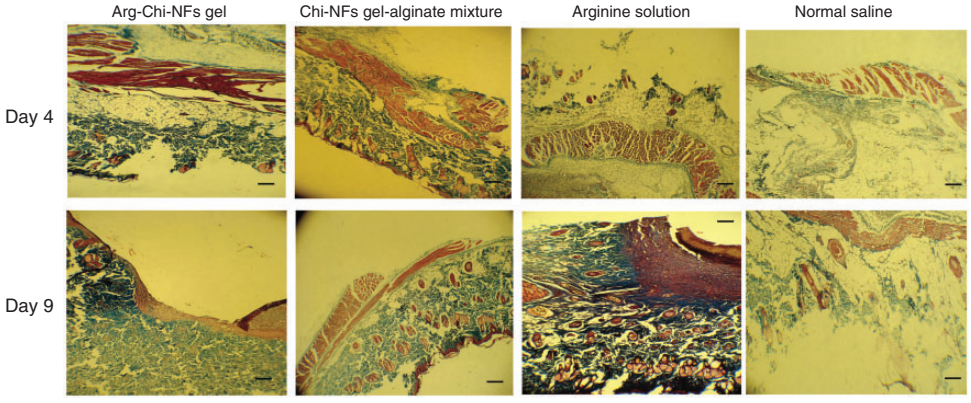


**Figure 7.** Micrographs of Masson's trichrome stained wounded tissue treated with Arg-Chi-NFs gel (F6), Chi-NFs gel (F5)–sodium alginate mixture, arginine solution, and normal saline at 4 and 9 days after. Magnification: 40×, scale bar, 75 μm. Resynthesized collagen fibers can be seen in blue color.

the entire of the wound was covered with new epithelium at the final of the study (Figure 6(a)). Conversely, the reepithelization score of both wounds that were treated with Chi-NFs gel (F5)–alginate mixture and arginine solution was approximately 2 and about a half of the wounds were covered by new epithelium after nine days in these groups. Less than a half of the wounds (score 1) that were treated with normal saline were covered by new epithelium after 9 days (Figure 6(a)). These results are consistent with the wound closure results. The Arg-Chi-NFs gel (F6) significantly improved the wound healing compared to both Chi-NFs gel-alginate mixture and arginine solution. Regarding the groups treated with the Chi-NFs gel (F5)–alginate mixture and arginine, solution presented a very similar reepithelialization, although the former had a slightly superior wound area reduction.

*Collagen deposition.* To evaluate the collagen deposition in the healed tissues, the skin wound sections were stained with Masson's trichrome, which revealed the extent of collagen deposition in healed tissue (Figure 7). On day 4, all groups presented a mild deposition of collagen from intensity of Masson's trichrome staining (blue) and according to the criteria set by Gal et al.[28] On day 9, in comparison with other groups, Arg-Chi-NFs gel treated group showed higher collagen deposition. There was no significant difference in the amount of collagen deposition in other groups (Figure 6(b)). The better collagen deposition in the Arg-Chi-NFs gel treated group might be attributed to the simultaneous use of arginine and Chi-NFs. Different studies showed that arginine and thereby NO can enhance collagen deposition in healed tissue in the final remodeling steps of wound healing.[49] Moreover, chitosan can increase collagen deposition

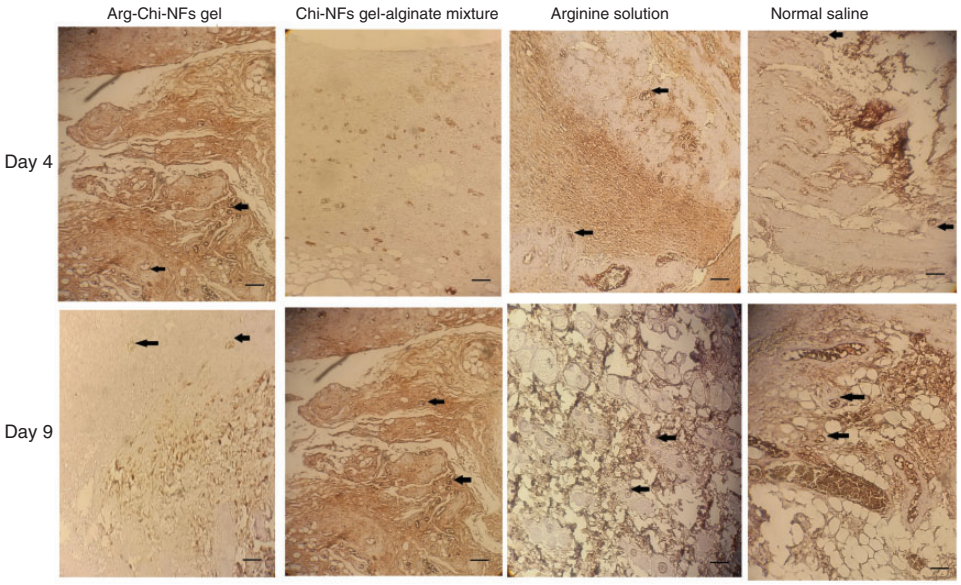


**Figure 8.** CD31-stained wound sections treated with Arg-Chi-NFs gel (F6), Chi-NFs gel (F5)–sodium alginate mixture, arginine solution, and normal saline at day 4 and 9 after wounding. The arrow shows the new vessels. Magnification: 100×, scale bar, 200 μm.

at the wound site by releasing N-acetyl-glucosamine monomers during the wound healing process.[50]

*Angiogenesis.* In addition to reepithelialization, arginine and thereby NO can promote angiogenesis during the proliferative phase. Angiogenesis or neovascularization is the hallmark of the proliferative phase in wound healing process and can restore the vascular integrity to the wound site.[1] Moreover, chitosan can also induce angiogenesis in the wound bed.[51] New vessel formation in wound tissues was evaluated by counting the newly formed blood vessels in wound tissue slides that immunohistochemically stained with anti CD31 monoclonal antibody that indicated the vascularization involved in angiogenesis (Figure 8).[52] The average new vessels counts across the whole wounds are shown in Figure 6(c). There was a significant increase in angiogenesis in wounds treated with Arg-Chi-NFs gel, compared to other groups after four days of surgery ($P$ value $< 0.05$). The group treated with Chi-NFs gel-alginate mixture showed considerable new vessel formation because of angiogenesis induction of chitosan.[19–20] These results demonstrate that arginine release from Arg-Chi-NFs gel is attributed to better angiogenesis compared to Chi-NFs gel–alginate mixture. Moreover, the ability of Chi-NFs gel to release arginine in a sustainable manner may be attributed to better wound healing effects (better reepithelization, more collagen deposition, and better angiogenesis) compared to the other groups.[22] At day 9, there was a decrease of CD 31 expressed cells in the wounds treated with Arg-Chi-NFs gel compared to other groups. The lesser angiogenesis on day 9 in Arg-Chi-NFs gel group is because of the complete wound healing in this group after nine days. On the other hand, other groups that are in the healing process phases still showed more angiogenesis (Figures 8 and 6(c)). In general, it was found that among different compounds, Arg-Chi-NFs gel was the most promising as it significantly improved wound healing process. Arg-Chi-NFs gel enhanced significantly the wound closure compared with other groups. In addition, the results of histological examination and immunohistochemical study showed that Arg-Chi-NFs gel showed higher reepithelialization and collagen deposition until the final day 9 and induced a significant angiogenesis on day 3 because of the effects of arginine and chitosan during the proliferative phase of wound healing.

## Conclusion

Herein, we report Arg-Chi-NFs gel may be an effective compound for the wound healing. Arg-Chi-NFs gel could be sterilized easily and used on the wound site. The suitable spreadability of Arg-Chi-NFs gel made the use of this gel easy at the wound site. Arg-Chi-NFs gel treatment was shown to enhance wound healing as evidenced by greater reepithelialization and angiogenesis and faster collagen synthesis when compared to other groups. Moreover, in vivo rat study confirmed treatment safety and efficacy of the excisional wound model. Collectively, these results suggested that Arg-chi-Nfs gel could be introduced as an effective wound dressing.

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