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Original Article

Effect of cell-free supernatant of *Bifidobacterium bifidum* combined with chitosan biodegradable film on full thickness wound healing in rats

Atena Bazjoo[1], Parvaneh Jafari[1*], Azam Marjani[2], Neda Akbari[1]

1. Department of Microbiology, Arak Branch, Islamic Azad University, Arak, Iran
2. Department of Chemistry, Arak Branch, Islamic Azad University, Arak, Iran

ABSTRACT

Introduction: Wound healing is one of the most critical issues human has been faced since the beginning of creation. Biodegradable polymers are of particular importance. In this study, cell-free supernatant (CFS) of *Bifidobacterium bifidum* combined with chitosan (CH) film was evaluated as a wound dressing.

Methods: Biodegradable films (CH and CFS/CH), as a novel wound dressing, were prepared. For the evaluation of dressing efficacy, 45 male Wistar rats weighing 200-250 g were randomly divided into 3 groups: negative control (without wound treatment), positive control (wound treatment by CH film), and probiotic (wound treatment by CFS/CH film). One full thickness wound was created on the dorsal area of the animals. The wound in positive control and probiotic groups were immediately covered by CH and CFS/CH dressing, respectively. Wound healing process was evaluated by macroscopic observation and histological analysis. During the treatment the expression of IL-1, TGF-B and IL-6 were assayed by qRT-PCR.

Results: Our results showed different infiltration patterns of macrophages, fibroblasts, and neutrophils in CFS/CH treated group. Enhanced disposition of collagen and elastin caused improvement of wound healing process by the film. Based on the gene expression results, use of CFS/CH film caused improvement in wound healing kinetic.

Conclusion: The biodegradable film based on chitosan and CFS of *B. bifidum* improves the wound healing process.

Keywords:
Wound healing
Chitosan
Cell-free supernatant
Biodegradable film
Cytokines

Introduction

The skin creates a protective barrier between the body and the outside environment, protecting the body against physical, chemical, and microbial damages as well as losing fluids. Therefore, it plays an important role in maintaining body homeostasis (Boer et al., 2016). Wound is any type of damage to the skin integrity that can be caused by various physical, chemical, and thermal factors. The intricate skin wound healing process consists of 4 sequential and overlapping stages:

* Corresponding author: Parvaneh Jafari, p-jafari@iau-arak.ac.ir
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hemostasis, inflammation, proliferation, and maturation or remodeling (Kasuya and Tokura, 2014).

The initial phase of homeostasis begins within the first few seconds after the ulcer creation and lasts up to one hour (Nami et al., 2016; Tamagawa-Mineoka, 2015). In this phase, activated cells release interleukins (such as TGF-β) which results in the recruitment of immune cells (e.g. neutrophils, monocytes, and immune cells) and initiation of inflammation phase (Gouin et al., 2017; McFadyen and Kaplan, 2015; Rosa and Fantozzi, 2013; Schmelz and Petersen, 2001) Neutrophils reach their maximum number in the wound site within 24 to 48 hours and produce IL-1, IL-6, TNF-α, and VEGF, which control the subsequent stages (Baum and Arpey, 2005). Then monocytes become the dominant cell population and some of them differentiate into macrophages (Sindrilaru and Scharffetter-Kochanek, 2013). Macrophages in collaboration with neutrophils, eventually orchestrate the inflammatory resolution (Ferrante and Leibovich, 2012; Jones et al., 2016). Fibroplasia, re-epithelialization, angiogenesis, and peripheral nerve repair are performed in the proliferation phase (Bao et al., 2009; Willenborg and Eming, 2014). Interleukins such as IL-1, TGF-β, INF-γ, and PDGF, control this phase (Greaves et al., 2013). At this stage, a large amount of collagen is produced by fibroblasts, replacing the early provisional matrix cross-linked with fibronectin (EPM) with collagen rich extracellular matrix (ECM) (Barker and Engler, 2017). In the remodeling phase, which can last for several months, collagen type III is replaced by type I and create a functional tissue (Maquart and Monboisse, 2014; Olczyk et al., 2014; Volk et al., 2011). It is well established that interleukins play an essential role in regulating different stages of wound healing and their expression has a temporal and spatial regulatory characteristic (Werner and Grose, 2003).

Any dysregulation of the wound healing process could result in complicated problems such as chronic wounds and even death. Nowadays, the treatment of wounds becomes a major challenge to healthcare systems worldwide. So, over the years different categories of wound care products such as dressing are introduced into the market. Dressings are classified based on their physical form, including ointment, film, foam, and gel. Transparent films protect the wound from bacterial invasion and accelerate healing process. Chitosan is the primary component used in the preparation of wound films. It has unique properties such as biodegradability, non-toxicity, antibacterial effects, and biocompatibility. Chitosan-based film stimulates the hemostasis phase of wound healing and accelerates tissue regeneration (Jones et al., 2006).

It is well established that probiotics and their metabolites can accelerate cutaneous wound healing by stimulating immune cells, inflammatory cell infiltration, and antibacterial effects. So, we decided to produce a new wound dressing film based on the chitosan, as a biodegradable polymer, and cell-free supernatant of *Bifidobacterium bifidum* as a probiotic strain. We evaluated the cutaneous wound healing properties of this novel film in a rat model.

Materials and methods

Preparation of cell-free supernatants (CFS)

Bifidobacterium bifidum ATCC 29521 was purchased from the Iranian Research Organization for Science and Technology (IROST). The safety and efficacy of the bacterial strain were evaluated according to WHO guidelines and it is used as a commercial probiotic. The MRS (Merck, Germany) medium supplemented with 0.5 g/L L-cysteine (Merck, Germany) was inoculated with 5% fresh bacterial overnight culture and incubated for 48 hr in an incubator containing 5% CO_2 at 37°C. The CFS was centrifuged at 6000 rpm for 30 min at 4°C. The CFS was filtered with a 0.2 µm filter and cultured in the MRS agar medium to ensure the absence of bacterial cells (Maghsood et al., 2018).

Preparation of biodegradable membrane

For preparing CH film, Chitosan powder (3.75%) was added to acidic double distilled water with pH=3.5 (using glacial acetic acid) (Clasen et al., 2006). The suspension was stirred on the stirrer for 2 hr at 30°C for complete dissolution. The solution was poured as a thin layer in preheated sterile glass containers for casting. After 24 hr incubation at 25°C, the polymer layer was removed by adding 80% ethanol for 30 min. A uniform film with a diameter of 0.2 mm was prepared and immersed in 0.1 M NaOH for 10 secand finally dried in the oven for 30 min at 40°C.

In order to prepare CFS/CH film, 62.5% supernatant solution was prepared in sterile distilled water and adjusted to pH=3.5 using acetic acid. The chitosan powder (3.75%) was added to the solution and treated for 2 hr on

the stirrer at 30°C. To provide a uniform and stable suspension, the solution was subjected to ultrasonic for half an hour and stirred again on the stirrer for 1 hr. Finally, the film was prepared as before.

Preparation and maintenance of rats

4-week old male Wistar rats (N=45), weighing 200-250 gwere obtained from Pasteur Institute of Iran. Rats were kept in separate cages at 23±2°C and in a 12 hr light/dark cycle with free access to water and standard food. After a 2-week acclimatization period, rats were randomly divided into three groups (n=15): negative control group (without wound treatment), positive control group (wound treatment by CH film), and probiotic group (wound treatment by CFS/CH film). All the interventions and experiments were performed according to the standard animal laboratories of Islamic Azad University, Arak Branch (ID: IR.IAU.ARAK.REC.1398.001).

Wound creation and treatment of rats

Rats were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) (Barreto et al., 2016). The hair on the dorsal part of the animal was shaved and the area was disinfected with 70% alcohol. One full-thickness wound was created in the posterior area of the animal, including epidermis and dermis at the dimensions of 1.3×1.3 cm^2. The CH and CFS/CH films were immersed in sterile water for a few seconds. The wet films were applied to the wound areas of the corresponding group immediately post wounding (PW). Using tape, the films were placed on the wound site and after 1 hr, the films were completely fixed by fluid absorption. It should be noted that the wounds were treated by films only once at day 0 PW. On days 1, 3, 7, 14, and 21 PW, three mice from each group were randomly selected and sacrificed according to the ethical guidelines. After the measurement of the wound area, specimen samples of tissue were collected from each rat, leaving a 5 mm margin of normal skin around the edges of the healed wound. Then, the wound healing process and neo-tissue formation were assayed by Histological analysis (H&E staining, Orcein and Masson's trichrome staining) and gene expression (IL-1, TGF-β, and IL-6).

Measurement of Wound contraction

The improvement of the healing process was evaluated by measuring the percentage of wound contraction. For this purpose, after anesthetizing rats on the aforementioned days, the wound dimensions were determined using a caliper and the percentage of healing was calculated based on the following formula:

Histological evaluation of tissues

The wound tissue samples were divided into two sections and used for histology and gene expression experiments. For histological analysis, tissue samples were fixed in 10% formalin for 24 hr. Tissue slides were prepared using a tissue processor. In brief, the sample was first dehydrated by ascending alcohol percentage. After preparing paraffin blocks, slides were made from tissue sections of 5 µm thickness (Dogan et al., 2009). After staining with H&E, tissue analysis was performed by evaluation of neutrophils, macrophages, fibroblasts, angiogenesis, and re-epithelialization in 20 fields of view.

Assessment of collagen and elastin synthesis

To evaluate the amount of collagen and elastin, tissue slides were prepared the same as before. Masson's trichrome staining was used to evaluate collagen. Briefly, after paraffin removal and rehydration in ethanol descending concentrations, the tissues were treated for 1 hr at 65°C in Bouin's solution. The slides were stained in Weigert's iron hematoxylin working solution for 10 min. Then they were stained in Biebrich scarlet-acid fuchsin solution (for 10 min), and phosphomolybdic-phosphotungstic acid solution (for 10 min), respectively. The slides were transferred to an aniline blue solution and stained for 10 min. Finally, dehydration was performed with 95% ethanol and absolute ethanol. A microscopic examination was performed after lamellation (Miller et al., 2017).

Orcein method was used to observe the elastic fibers. After paraffin removal, the slides were stained with orcein. After acid-alcohol decolorization, re-staining was performed using safranine. Finally, alcohol dehydration and xylazine clearance were performed. Elastin deposition was monitored using a light microscope (Kornhauser, 1952).

Evaluation of IL-1, IL-6 and TGF-β genes expression

The expression of genes including IL-1(GenBank accession no. D00403), IL-6 (GenBank accession no. E02522), and TGF-β (GenBank accession no. NM-021578) were determined by RT-PCR. The β-actin gene

(GenBank accession no. V01217 J00691) was used as an internal control. Briefly, the total RNA was isolated using universal purification kit (EURX Poland) according to the manufacturer's instructions. PrimeScript RT reagent kit (Takara Bio, Ohtsu, Japan) was used for cDNA synthesis. Quantitative RT-PCR was conducted using the rotor gene 6000 Corbette detection system and SYBR Premix Ex Taq (Takara Bio). Thermal cycling included initial activation for 2 min at 95°C, 45 cycles at 60°C for 15 sec, and final extension at 72°C for 15 sec (Nouri et al., 2016). The forward and reverse primers sequences were as follows:

IL-1: 5'-TCTTTGAAGAAGAGCCCGTCC-3' and 5'- GGTCGTCATCATCCCACGAG-3'

IL-6: 5'- CGAAAGTCAACTCCATCTGCC-3 and 5'- GGCAACTGGCTGGAAGTCTCT-3'

TGF-β: 5'- CCTGGATACCAACTATTGCTTCAG and 5'- CAGACAGAAGTTGGCATGGTAG -3'

β-actin: 5'- AGAGCTATGAGCTGCCTGACG-3' and 'CTGCATCCGGTCAGCGATAC-3'

The PCR specificity was measured by melting curve analysis and electrophoresing on 2% agarose gel. The mRNA expression level was normalized to β-actin expression and expressed as ΔCT value ((ΔCT = CT B-actin - Ct target mRNA). The relative expression levels of the calibrator were determined using $R = 2^{-\Delta Ct}$

Data analysis

Data were analyzed by ANOVA followed by Tukey test using Graphpad Prism 8 software. All data are presented as mean ± SEM and P-value less than 0.05 was considered significant.

Results

Wound contraction

Wound dimensions were measured using a caliper and the percentages of wound healing were recorded on different days (Table 1 and Figure 1). Wound closure is one of the first signs of wound healing that occurs by re-epithelialization.

In the CFS/CH group, the percentage of wound healing was significantly better than other groups on all days. In this group, the wound was completely healed on day 14 PW which was significantly higher than Ctrl- and

TABLE 1: Mean and standard error of mean of wound healing percentage in rat groups. Negative group with no treatment, CH group with chitosan film treatment, and CFS/CH group with cell-free supernatant/chitosan film treatment.

Day (PW)	Treatment groups (Mean±SEM)		
	Ctrl-	CH	CFS/CH
1	8.75± 0.41^{c}	22.25 ±1.99^{b}	31.63±1.43^{a}
3	30.75±1.37^{c}	48.13±0.40^{b}	56.00±1.18^{a}
7	56.67±0.84^{c}	74.08±0.71^{b}	83.83±0.70^{a}
14	78.58±1.53^{c}	94.75±1.31^{b}	100%
16	85.74±1.04^{b}	99.33±0.22^{a}	100%
21	95.62±0.72	100%	100%

Common letters in a row indicate no significant difference

TABLE 2: Infiltration of neutrophils, macrophages, and fibroblasts into the wound site of different groups. Negative group with no treatment, chitosan group with chitosan film treatment, and probiotic group with CFS/chitosan film treatment

Day	Mean ±SEM								
	Neutrophil			Macrophage			Fibroblast		
	Ctrl-	CH	CFS/CH	Ctrl-	CH	CFS/CH	Ctrl-	CH	CFS/CH
1	7.42±1.64^{c}	12.79±1.91^{b}	17.73±2.01^{a}	2.46±0.68^{b}	6.41±078^{a}	9.27±0.49^{a}	1.02±0.38^{b}	2.32±1.31^{a}	2.85±1.91^{a}
3	16.71±1.11^{a}	9.57± 0.65^{b}	5.14±0.40^{c}	3.29±0.52^{c}	10.14±0.74^{b}	14.57±0.65^{a}	2.14±0.40^{c}	3.71±0.56^{b}	5.14±0.51^{b}
7	7.86±0.86^{a}	3.29± 0.42^{b}	2.29± 0.52^{b}	18.14±0.59^{a}	5.14±0.26^{b}	3.86±0.63^{b}	3.15±0.46^{b}	17.86±0.55^{a}	19.43±0.61^{a}
14	5.58±0.78^{a}	2.29±0.36^{b}	1.43±0.30^{b}	10.29±0.68^{a}	3.29±0.29^{b}	2.14±0.55^{b}	10.14±0.60^{a}	7.71±0.68^{b}	4.29±0.52^{c}
21	3.43± 0.43^{a}	2.14±0.34^{b}	1.29±0.29^{b}	4.58±0.37^{a}	2.29±0.42^{b}	1.28±0.36^{b}	7.14±0.26^{a}	5.43±0.37^{b}	3.00±0.58^{c}

Common letters in a row indicate no significant difference

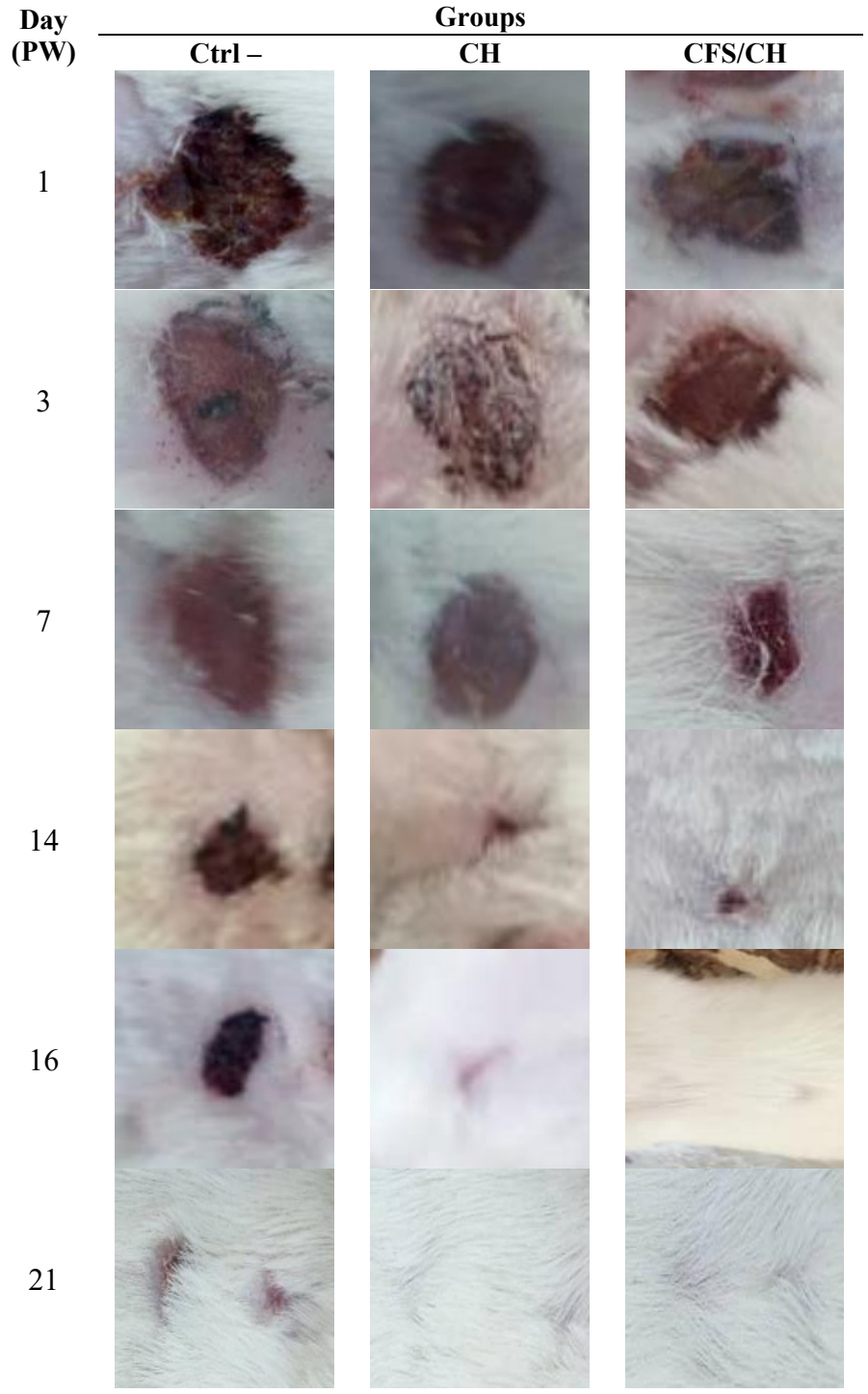

FIGURE 1. Wound images on different days post wounding in groups. (Ctrl-): the group with no treatment, (CH): the group treated with chitosan film, and (CFS/CH): the group treated with cell free supernatant/chitosan film.

CH groups (P<0.0001 and P=0.0072, respectively). The chitosan film dressing also improved the wound healing process compared to the Ctrl- group during the experiment. The wound was almost healed in this group on day 16 PW (99.33% healing), which was significantly more than Ctrl- group (P=0.0060). Wound healing in the Ctrl- group was 95% on day 21 PW, indicating a slow healing process compared to the other groups.

Histological evaluation of tissues

Histological evaluation of the wound healing process was done by H&E staining of tissue slides on different

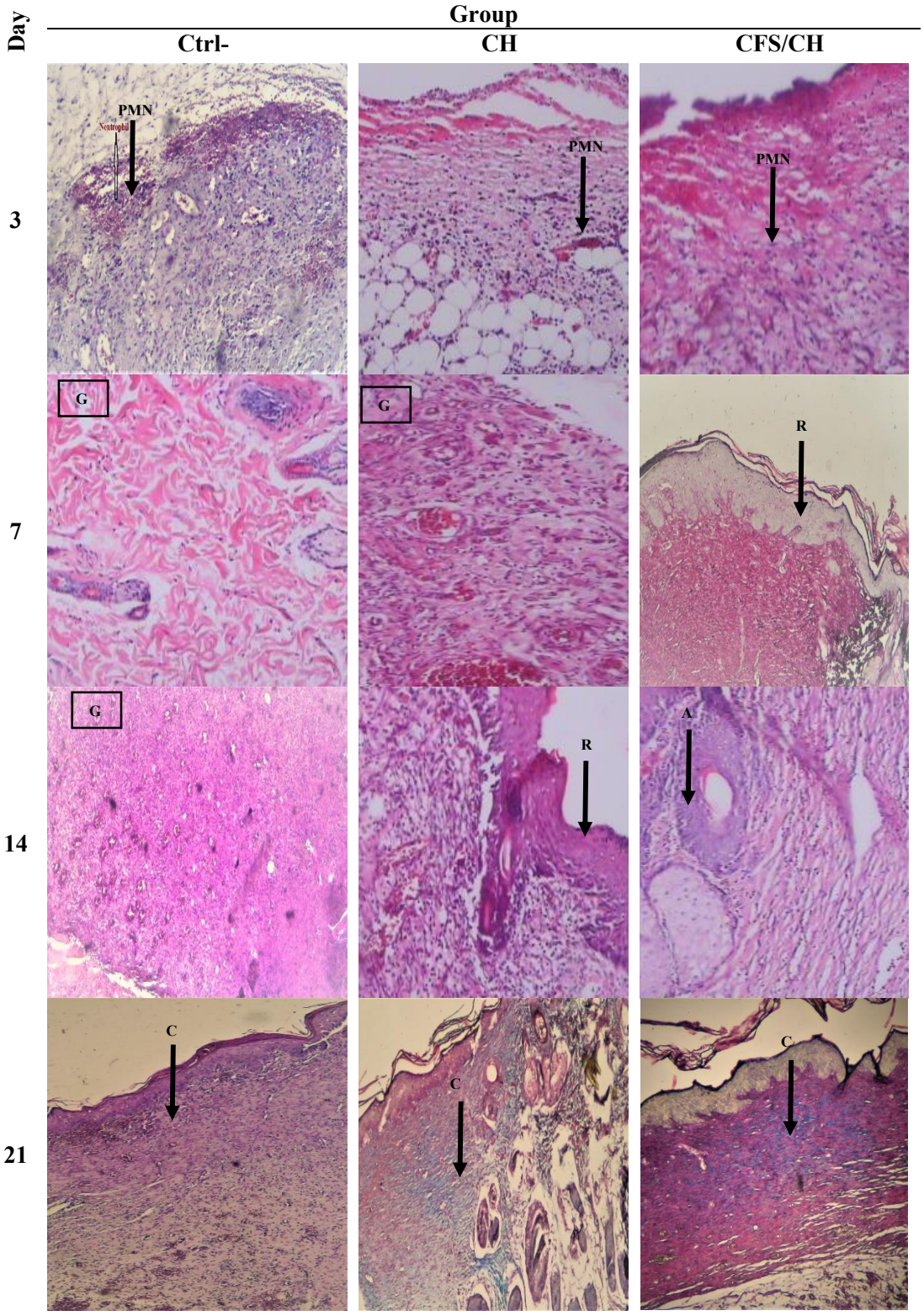

FIGURE 2. Histological images of wound sections in different groups stained with H & E. PMN: Polymorphonuclear cells, G: Granulation tissue, R: Reepithelization, A: Angiogenesis, And C: Collagen
(Ctrl-): the group with no treatment, (CH): the group treated with chitosan film, and (CFS/CH): the group treated with cell free supernatant/ chitosan film.

days. Neutrophils, macrophages, and fibroblasts are the most important cells involved in the wound healing process, which were counted in 20 fields of view (Table 2 and Figure 2). The results showed that the neutrophile

TABLE 3: Collagen and elastin fibers deposition in different groups. Negative group with no treatment, chitosan group with chitosan film treatment, and probiotic group with CFS/chitosan film treatment

Day	Mean ± SEM					
	Collagen			Elastin		
	Ctrl-	CH	CFS/CH	Ctrl-	CH	CFS/CH
3	0.039±0.014^{a}	0.067±0.017^{a}	0.077±0.013^{a}	0.067±0.017^{b}	0.436±0.154^{a}	0.736±0.195^{a}
7	0.067±0.024^{b}	0.086±0.014^{b}	0.117±0.007^{a}	0.137±0.016^{c}	1.071±0.028^{b}	1.471±0.115^{a}
14	0.074±0.019^{c}	0.743±0.092^{b}	1.100±0.048^{a}	0.486±0.137^{c}	1.343±0.057^{b}	1.871±0.084^{a}
21	1.171±0.089^{b}	1.343±0.134^{b}	2.186±0.070^{a}	1.057±0.030^{c}	2.171±0.068^{b}	2.829±0.099^{a}

Common letters in a row indicate no significant difference

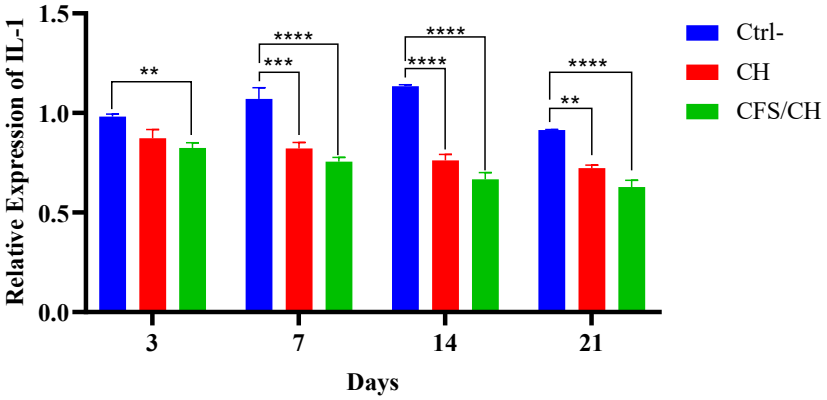

FIGURE 3. Relative levels of IL-1 expression on different treatment days.
(Ctrl-): the group with no treatment, (CH): the group treated with chitosan film, and (CFS/CH): the group treated with cell free supernatant/chitosan film. *p* values were regarded as significantly different at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

reached the maximum number in the CH and CFS/CH groups on day 1 PW and then decreased. Interestingly, this number was significantly higher in the CFS/CH group than the CH group (P=0.011). In the Ctrl- group, the peak of neutrophiles was on day 3 PW which indicates faster onset of inflammation in the CFS/CH and CH groups.

Macrophages are the second cell group which enter the wound site during the inflammatory phase. The results showed that the number of these cells on the first day in the treatment groups was significantly higher than the Ctrl- group. In the following days, the number of macrophages increased in all three groups. In the film-treated groups, the peak of macrophages was on day 3 PW. Obviously, this number was significantly higher in CFS/CH group than the CH group (P=0.0007). In the Ctrl- group, massive infiltration of macrophages was observed on day 7 PW, which indicated severe and longer phase of inflammation.

Fibroblasts are the dominant cells during the proliferation phase. The results showed that in the two film-treated groups, the maximum number of these cells was on day 7 PW without any significant difference between them. In subsequent days, significant decrement was observed in fibroblasts in the two groups. In the Ctrl- group, the peak of fibroblasts was on day 14 PW then, decreased significantly. The decrease in the number of fibroblasts at the site of the wound indicates the onset of the maturation phase and the transformation of granulation tissue into normal tissue.

Evaluation of tissue firmness based on collagen and elastin synthesis

Collagen synthesis by fibroblasts is one of the major causes of tissue firmness and wound healing that occurs during the proliferation phase. The results of the evaluation of collagen and elastin are indicated in Table 3 and Figure 2. The accumulation of collagen fiber gradually increased in all groups. Surprisingly, in the CFS/CH group, the deposition of this fiber was enhanced by the application of cell-free supernatant of the probiotic strain. The same accumulation pattern of elastin fibers was observed, too. The CFS/CH film caused induction in elastin synthesis.

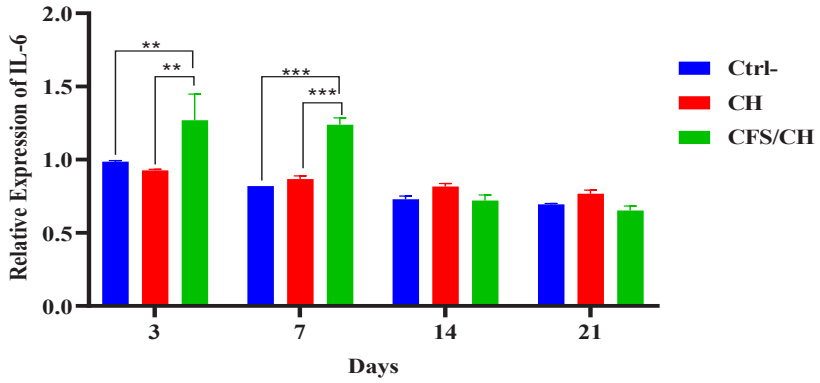

FIGURE 4. Relative levels of IL-6 expression on different treatment days. (Ctrl-): the group with no treatment, (CH): the group treated with chitosan film, and (CFS/CH): the group treated with cell free supernatant/chitosan film. *p* values were regarded as significantly different at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

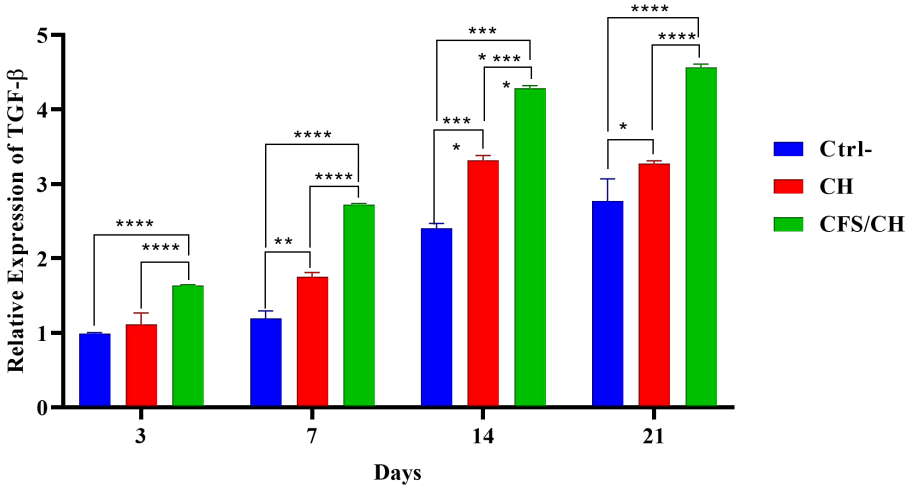

FIGURE 5. Relative levels of TGF-β expression on different treatment days.
(Ctrl-): the group with no treatment, (CH): the group treated with chitosan film, and (CFS/CH): the group treated with cell free supernatant/chitosan film. *p* values were regarded as significantly different at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

IL-1, IL-6 and TGF-β genes expression

The results showed that the expression of IL-1, as a proinflammatory, in the CFS/CH and CH groups was significantly lower than Ctrl- group during the experiment (Figure 3). On days 7 and 7 PW, the concentration of IL-6 in the CFS/CH group was significantly higher than the other groups. As the wound healing progressed, there was no significant difference in IL-6 expression levels between groups (Figure 4). TGF-β is a multifunctional cytokine which its expression in the CFS/CH group was significantly higher than the other groups on all days (Figure 5).

Discussion

The intricate skin wound repair process is an inter-reliable and overlapped process that is orchestrated by cells, chemokines, and chemotactic agents. Any abnormality in this process can lead to problems such as fibrosis, chronic ulcers, disability, or even death. Therefore, research in the field of accelerating wound healing products such as new wound dressing has gained special importance.

One of the most important components used in dressings film is biodegradable chitosan. Chitosan is composed of N-acetyl glucosamine units that can improve wound healing through similarity to hyaluronic acid in the extracellular matrix (Keong and Halim, 2009). Most importantly, chitosan can cause analgesia due to its cool, pleasant, and soothing effects. Also, it may prevent chronic inflammation in ulcers by reducing the secretion of proinflammatory cytokines (Ahmed and Ikram, 2016).

B. bifidum is one of the most important human probiotics, which modulate the immune system. This bacterium

can alter gene expression in intestinal epithelial cells by interkingdom quorum sensing and may play a role in reducing inflammation, too. Extensive research have been showed that bacterial metabolites (e. g, proteins, nucleic acids, short-chain fatty acids, etc.) influence the expression of nuclear factors and genes (Ramos et al., 2015). So, we decided to design a new biodegradable film with chitosan and CFS of *B. bifidum* and evaluate it in an excisional wound model in male Wistar rats. Free amine groups in the chitosan structure bond with proteins, amino acids, nucleic acids, fatty acids, and phospholipids of CFS and provide a coherent composite structure. To our knowledge, this is the first report of producing a dressing film using biodegradable polymer combined with cell-free supernatant of a probiotic strain.

During wound healing, platelets form a clot and initiate the homeostasis phase. They promote the subsequent phases of wound healing by the release of chemokines and chemoattractants. Platelets produce various interleukins, of which TGF-β, PDGF, VEGF, and FGF are the most important (Guo and DiPietro, 2010). TGF-β is a multifunctional cytokine and causes infiltration of immune cells (neutrophils, monocytes, and macrophages), proliferation and differentiation of fibroblasts, ECM deposition, angiogenesis, and wound tissue firmness (Pakyari et al., 2013). The results of this study showed that by CFS/CH film, TGF-β expression increased throughout the test period compared to the other groups. This phenomenon caused an increase in neutrophil and macrophage infiltration. The maximum infiltration of neutrophils in the CFS/CH group was on day 1 PW which was 58.15% and 27.86% higher than Ctrl- and CH groups, respectively. Also, in the CFS/CH group, the peak of macrophage infiltration was observed on day 3 PW which was 73.46% and 30.85% greater than the Ctrl- and CH groups, respectively. Creating a powerful and rapid inflammation phase in CFS/CH group accelerated the wound healing process and prevented wound infection, as these alien cells play an important role in removing the remnants of damaged cells and pathogens in the wound site based on damage-associated molecular pattern (DAMP) and pathogen-associated molecular pattern (PAMP). On the other hand, these cells orchestrate subsequent phases of wound healing, from proliferation to final maturation by secreting chemokines and interleukins such as IL-1 and IL-6.

IL-1 is produced during the early phase of wound healing and is capable of inducing a variety of growth factors and chemokines involved in the acute inflammation phase. Even the use of topical ointments containing this interleukin accelerates the wound healing process. IL-6 is a keratinocytes mitogen factor and attracts neutrophils, therefore playing a role in the progression of the inflammation phase and migration of keratinocytes and consequently re-epithelialization.

In the CFS/CH group, after day 3 PW the IL-1 expression level decreased. It was accompanied by a reduction in the number of inflammatory cells and faster onset of the proliferation and maturation phase. Interestingly, CFS/CH membrane caused an increase in IL-6 expression during the first 7 days of healing. This interleukin accelerates re-epithelialization and faster wound healing. So, after 14 days the wound completely closed in CFS/CH group. It seems that this membrane increases IL-6 expression and leads to faster and more efficient migration of keratinocytes from the wound margin and wound closure.

Chitosan has been shown to decrease the expression of IL-6 and IL-1 pro-inflammatory cytokines in macrophages by blocking the MAPK pathway and inhibiting the activation of nuclear factor kappa (NF-κB) and activator protein-1 (AP1) (Ma et al., 2011). This study showed that in the CFS/CH group, the IL-1 expression decreased compared to the Ctrl-, even though the number of macrophages and neutrophils increased. This may be due to the anti-inflammatory effects of chitosan. Interestingly, chitosan alone did not affect the IL-6 expression level, while the probiotic membrane increased the expression of this cytokine.

What is important during the wound healing is decreasing the number of macrophages because continued inflammation can lead to chronic wounds with many medical problems. Increased secretion of proinflammatory cytokines delays the onset of the proliferative phase and subsequently delays angiogenesis and tissue remodeling. In the CFS/CH film group, the inflammatory phase was observed within the first 2 to 3 days PW and then diminished.

As it was told, TGF-β is a multifunctional growth factor which causes keratinocytes migration in the fibronectin-rich provisional matrix by increasing the expression of integrins, but its stronger and more stable expression induces hypertrophic scars. Our results showed that in the CFS/CH group the expression of this interleukin was

higher than the other groups during the whole test period. Contrary to the expectation, the hypertrophic scar was not seen in the CFS/CH group. This could be due to the decrease or increase in IL-1 and IL-6 levels respectively.

Rationally, as the TGF-β level increases in CFS/CH group, fibroplasia is accelerated and aggravated. It should be noted that although the TGF-β level was higher in the probiotic group compared to the chitosan film, the number of fibroblasts in these two groups was not significantly different. This may be due to the low accuracy of the cell counting method in histological tests. Also, it can be related to increased TGF-β expression which is a consequence of enhanced transcription of the relevant genes in the probiotic group.

Studies have shown that chitosan can increase the synthesis of hydroxyproline and consequently collagen deposition in tissues (Abbaszadeh et al., 2019). Our results showed that at the end of the 21-day PW, there was no significant difference in the amount of collagen deposition in the Ctrl- and CH groups. In the CFS/CH group, the increase in collagen synthesis was more than 80% compared to these groups. Elastin fibers also increased 73.46% and 30.85% in the CFS/CH group compared to the Ctrl- and CH groups, respectively. This was expected given the role of TGF-β in fibroplasia and increased collagen synthesis. Increased collagen formation was visible with increasing wound firmness in the CFS/CH group. This result was in line with the results of Salaran et al., 2019, which showed that *Lactobacillus plantarum* increased fibroblast synthesis and subsequently stimulated collagen synthesis and deposition by increasing the expression of anti-inflammatory cytokines such as TGF-β (Salaran et al., 2019).

Conclusion

The results of this study showed that chitosan as a suitable scaffold can be used to make probiotic cell-free supernatant-based dressing film. The film designed in this study enhanced and accelerated the inflammation phase by increasing the inflammatory cells infiltration such as neutrophils and macrophages. This phase is the most important phase of wound healing and coordinates the other phases. An increase in the number of these cells and the expression of proinflammatory interleukins and growth factors such as IL-6 and TGF-β accelerated fibroplasia(Christmann et al., 2013). Subsequently, extracellular matrix production, collagenization, and re-epithelialization were expanded (Ballotta et al., 2014). Finally, it can be concluded that this dressing can be suggested as a suitable wound dressing to improve the wound healing process.

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Conflict of interest

The authors declared no conflict of interest regarding the publication of this paper.

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