Research Article

The Effect of Cartilage and Bacteria-derived Glycoproteins as a Biological Dressing on Wound Healing

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Abstract

Immediate intervention with minimal side effects is the most significant factor in the enhancement of wound healing. However, a majority of drugs used for this purpose are chemical-based containing various compounds, such as sulfite, which sometimes causes allergic reactions in a number of patients, or anti-inflammatory agents that cause elevated blood sugar and weight gain. Hence, many researchers look for natural compounds, such as glycoproteins, not only to reduce the side effects but also to improve the speed of healing. In this study, we have created a natural biological dressing using the combination of extracellular matrix (ECM) derived from articular cartilage and DH5α bacterial ghost (BG). Both articular cartilage and BG contain high amounts of collagen and glycoproteins, and proteoglycans, respectively. The experimental wound on the rabbit pinna was treated by the biological dressing. Then microbial, scanning electron microscopy and microscopic analyses measured the wound healing parameters, including the number of fibroblast cells, the collagen contents, percentage of wound closure, and the number of colonies. The results confirmed ECM (OC), BG (OG) and their mixture (OGC) groups have better effects than control groups. Histological parameters, such as number of fibroblast cells and the amount of collagen fibers, represented a greater degree of wound healing in OGC group compared with OC, OG, and control groups. Our findings proved that ECM and bacterial ghost effectively increased the rate of wound healing. The mixture of ECM and BG provides a biological dressing that could be used in wound repair in the future.

***Keywords*:** Decellularization; Biological dressing; Bovine articular cartilage; Extracellular matrix; Bacterial ghost

Introduction

Chronic wounds are considered as a health burden and may cause physical disability and infectious diseases; so, rapid treatment of injuries with minimum adverse effects is urgent (Caporusso et al. 2019). Failure in wound healing can lead to microbial infection, increased morbidity, and imposed financial hardship, and emotional stress on health systems (Brigham and McLoughlin 1996). However, our knowledge about the wound healing process has markedly advanced in recent years. Wound healing is a critical process for maintaining tissue homeostasis leading to restitution of tissue integrity and barrier function (Hackam and Ford 2002). The healing process reflects the interactions among different cell types, growth factors, cytokines, blood components, and the ECM. The process of wound healing is complex in mammals, including three interrelated phases, namely inflammation, proliferation, and tissue remodeling (Greaves et al. 2013).

The first phase is inflammation, which begins shortly after the injury. In this phase, macrophages and neutrophils are recruited to protect the site of injury against invasive microbes. Also, capillaries, blood platelets, and active cytokines are increased. In the proliferation phase that occurs 1 to 3 weeks after a wound in humans, angiogenesis is a hallmark characterized by the increased proliferation of fibroblasts and re-epithelialization. The third phase is tissue remodeling, in which fibroblasts produce collagen by stimulating macrophages. At this phase, the synthesis and accumulation of collagen are propagated, and other cellular matrix proteins improve the strength and integrity of the damaged area (Broughton et al. 2006; Gantwerker and Hom 2011).

Due to the increased prevalence of wounds and its impact on high-cost treatment, the need for effective, safe, and affordable wound healing promoters would be needed.

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Alternative or complementary medicine has been extensively used for wound healing. From the past to the present, natural compounds, such as plant-derived materials and naturally occurring compounds such as honey, as an alternative and complementary medicine, are used for wound healing (Agyare et al. 2019). Although biological wound dressings, including natural-derived substances, have long been employed for wound healing, clinical trials are still in their early stages. These products have many advantages, such as possessing antioxidant, anti-inflammatory, antimicrobial agents, as well as enhancers for re-epithelialization and collagen synthesis (Ibrahim et al. 2018).

Cell-secreted compounds, including proteins and glycoproteins, play a positive role in wound healing. In multicellular organisms, the extracellular matrix (ECM) is considered a rich source of these compounds. ECM is composed of glycosaminoglycans (GAGs), proteoglycans (PGs), and collagens (Ghatak et al. 2015). ECM plays a physical role in tissue maintenance and acts as scaffold support for tissues. It can also control cellular signaling and behavior, such as proliferation, differentiation, and migration during the complex wound healing process. The surface macromolecules of bacteria are another source of glycoproteins. These compositions can be obtained using the bacterial ghost technique (Paukner et al. 2006; Kawano et al. 2021).

This study aims to assay the wound healing potential of biological dressing that was composed of ECM derived from decellularized bovine articular cartilage and bacterial ghost in the form of ointment in wound healing. In this study, wound healing parameters showed that compounds obtained from ECM and bacterial ghost could expedite the healing process in an experimental wound created on the rabbit pinna.

Materials and Methods

Isolation of ECM derived from Decellularized Cartilage

The entire experimental procedures carried out were approved by the Animal Research Ethics Committee of the Ferdowsi University of Mashhad (ethics code: IR.UM.REC.1398.122). In this experimental study, bovine articular cartilage was harvested from the sacrificed animals immediately after slaughtering. The articular cartilage was stored at -4°C before decellularization. Frozen samples were then thawed at room temperature and washed with sterile phosphate-buffered saline (PBS); these freeze-thaw cycles were repeated five times. In the next step, samples were then incubated with 0.25% trypsin at 37°C for 1 hour. After that, specimens were rinsed in 5% SDS and 2.5% Triton x-100 (Merck, Germany) solutions for 3 hours, followed by gentle agitation to achieve maximum elimination of cell debris while maintaining the ECM compositions. Eventually, in order to remove residual detergents from ECM derived from articular cartilage, samples were washed with sterile distilled water, 70% ethanol, and sterile PBS in a shaker incubator.

Histological Analyses

Specimens were fixed in Bouin's solution, dehydrated through a graded series of ethanol, embedded in paraffin (Lab-O-Wax, Italy), cross-sectioned at a thickness of 7 µm by a microtome apparatus (Leits, Austria), deparaffinized by xylene, rehydrated, and finally stained with hematoxylin and eosin (H&E) to determine the construct cellularity. Toluidine blue (Merck, Darmstadt, Germany) staining was performed to qualitatively measure the amount of GAGs in ECM derived from decellularized cartilage. The sections were observed under a polarizing microscope (Olympus, IX70, Japan) stained with Picrosirius red (Merck, Darmstadt, Germany) served as a specific stain to identify the collagen structure. The DNA content of ECM derived from decellularized cartilage was determined by a fluorescent dye, 4, 6 diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Taufkirchen, Germany) (Kapuscinski 1995).

Wound Microbiology

Wound microbiology was conducted to analyze the effect of biological dressing on wound bacterial burden. Samples were obtained from the wound region with a swab on days 0, 3, 7, 10, 15, and 21 after the creation of injury. Afterward, samples were cultured on the agar medium and incubated at 37 °C for 24 hours. Finally, the resulting colonies were counted (Park et al. 2008).

Generation of Bacterial Ghost

The pmET32c plasmid was previously generated at our laboratory (Soleymani et al. 2020). The *E. coli* DH5α (was generously donated by Research Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran) was transfected by the pmET32c plasmid, and bacterial ghosts were prepared as previously described. Briefly, to generate the *E. coli* DH5α bacterial ghost, the expression of E-lysis and staphylococcal nuclease (SNUC) genes were induced by the thermal inactivation of λ repressor at 42 °C and the addition

of isopropyl β- d-1-thiogalactopyranoside (IPTG) to the culture medium, respectively. For the production of BG, a single transfected colony was added to the SOB^{++} medium and cultured overnight in a shaker incubator at 28 °C temperature. After that, 1 ml of the culture medium was used to inoculate 200 mL of SOB^{++} medium and then incubated at 28 °C with shaking (150 rpm) to achieve an optical density at 600 nm (OD_{600nm}). IPTG (5 mM) was added to the culture medium (OD_{600nm} of 0.2–0.3) to induce SNUC expression. After 45 min, with an increase in the temperature from 28 to 42 °C, the expression of E-lysis was induced. The culture was monitored for 4 hours at a wavelength of 600 nm. Finally, the *E. coli* DH5α bacterial ghost was centrifuged, washed three times with 0.9% NaCl solution, and finally stored at 80 °C for subsequent analyses.

Preparation of Biological Dressings

The ointment base consisted of two oil and aqueous phases were prepared as previously described by Mashreghi et al (Mashreghi et al. 2013). Briefly, the biological dressing containing bacterial ghost and ECM derived from cartilage was prepared by pulverizing the ECM using a Mixer Mill device and lyophilizing the bacterial ghost. Next, the resulting mixture was added to the ointment base and mixed.

Animal Handling, Induction of Experimental Wound Model, and Histological Evaluations

In this study, 12 male New Zealand white rabbits with an approximate weight of 2,500 gr and an age range of 2–3 months were purchased from the Razi Institute of Mashhad, Iran. We created an experimental wound on the rabbit pinna using a punching apparatus. For this purpose, the hairs of the ear pinna were shaved and sterilized with ethanol (70%). Afterward, by means of lidocaine spray, the entire surface of the ears was locally anesthetized. After that, 3 holes with a diameter of 2 mm were created in each pinna at the medial region of the ears, located between peripheral veins and central arteries. The ointment base was applied for the O group, a mixture of BG and the ointment base for the OG group, a mixture of ECM and the ointment base for the OC group, a mixture of ECM and bacterial ghost, as well as the ointment base for the OCG group. A swab saturated with topical ointment was rubbed on each wound, while the control group (C) was left untreated.

Wound closure was analyzed in each group using images taken by a digital camera. Then, to evaluate the wound healing parameters, sampling was performed at 0, 3, 7, 10, 15, and 21 days post-injury (Schallberger et al. 2008). The animals were sacrificed, and the wound region and surrounded parts were isolated with a scalpel. The obtained specimens were fixed in Bouin's solution. The number of fibroblast cells was determined by H&E staining. For each wound region, four sections were specified, and three regions in each section were selected to be visualized at 100X magnitude. Masson's trichrome staining was utilized to detect collagen fibers, resulting in blue-colored fibers that contain collagen. The stained regions are ranked based on the color intensity as follows 1: very rare, 2: rarely, 3: moderate, 4: very, and 5: very much.

Scanning Electron Microscopy (SEM)

In order to prepare DH5α BG for electron microscopy, samples were fixed in 2.5% glutaraldehyde (TAAB Laboratories, UK) for 24 h, followed by three 15-min washing steps in 0.1 M sodium cacodylate buffer (pH 7.4, TAAB Laboratories, UK). Next, samples were treated with 1% osmium tetroxide (TAAB Laboratories, UK) for 1 h, rinsed again in 0.1 M sodium cacodylate buffer, and dehydrated through a graded series of ethanol. Finally, samples were fixed on metal stubs coated with gold-palladium by the sputtering method (Sputter coater, SC7620, East Sussex, UK). Consequently, specimens were observed using SEM (LEO 1450VP, Germany).

Statistical Analysis

The variables included the percentage of wound closure, the number of colonies, the number of fibroblast cells, the collagen contents, and the number of colonies. Statistical analyses were performed using GraphPad Prism software (8.0) . Kruskal–Wallis test followed by Dunn's multiple comparisons analysis was used to find statistically significant differences. Differences were considered significant when the p value was ≤ 0.05. The significant differences between the C group and the other groups are shown as $^{****}p \leq 0.0001$, $^{***}p \leq 0.001$, $^{**}p \leq 0.01$ and $^{*}p \leq 0.05$.

Results

Histological Examination of ECM derived from Bovine Articular Cartilage

Articular cartilage was decellularized with enzymatic and physicochemical experiments using 1) 0.25% trypsin, 2) snap freeze-thaw, and 3) treatment with detergents.

As shown in Figure 1, decellularized cartilage is devoid of chondrocytes compared with naïve cartilage. Also, toluidine blue and Picrosirius-red

staining methods demonstrated GAGs. Also, collagen-rich contents remained intact in the ECM. Therefore, the nuclei of cartilage stained with DAPI are visualized as bright spots and indicate the presence of chondrocytes in the cartilage matrix (Figure 1). However, the image of decellularized cartilage exhibits the matrix completely dark due to the absence of any nucleus.

BG Generation

In order to assess the generation of BG, the samples were analyzed by SEM (Figure 2). The surface morphological structure of *E. coli* bacterial ghost indicates the number of pores, implying the efficiency of the lysis process.

ECM derived from bovine articular cartilage as well as BG were ground and mixed with the ointment base. The resulting mixture was utilized as a biological dressing to alleviate the pinna in a rabbit model (Figure 3).

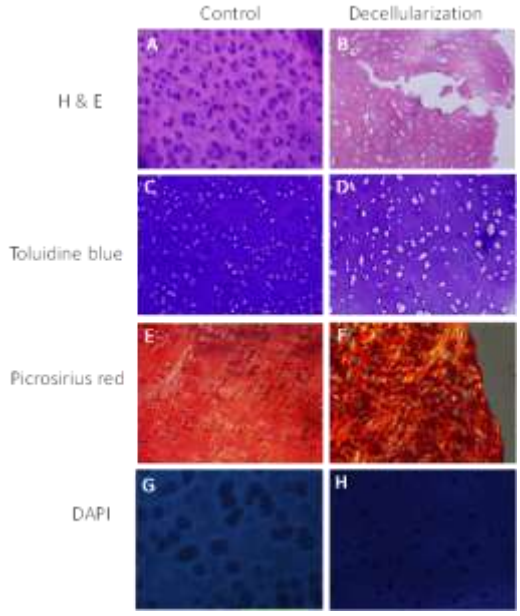

Figure 1. Control and decellularized articular cartilage samples. A & B) H&E staining demonstrated that treatment with trypsin and physicochemical methods led to the complete removal of chondrocytes. Toluidine blue and Picrosirius Red staining methods displayed the intactness of GAGs and collagen contents (C-F). Bovine articular cartilage is stained with DAPI before and after the decellularization process (G and H) (100X magnification).

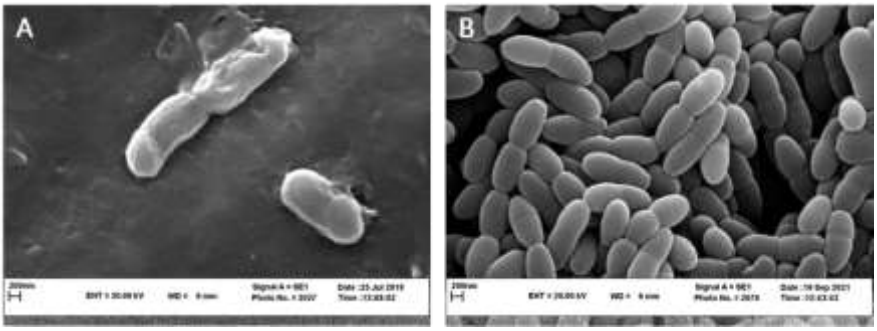

Figure 2. SEM of bacterial ghost. A) *E. coli* bacteria. B) After BG production process, the cytoplasmic contents are released through the pores.

Figure 3. Preparation of biological depressing; A) Decellularized bovine articular cartilage, B) ECM derived from bovine articular cartilage, C) Biological dressing that contains ECM derived from bovine articular cartilage and BG mixed with the ointment base.

Wound closure

In order to evaluate the wound healing ability of biological depressing, the percentage of wound closure was measured on different days through monitoring the wound region and comparison with control wound size. On day 3, there was a significant difference in the percentage of wound closure when OG, OC, and OGC groups were compared with the control groups (C and O). However, on days 7 and 10 post-injury, the differences between the treatment and control groups were not statistically significant. On day 15 post-injury, the differences in the percentage of wound closure were significantly increased in the OGC compared with other groups. On day 21 post-injury, the process of wound closure was completed in OGC in comparison to other groups (Figure. 4). On this day, OG and OC groups had higher degrees of wound closure than control groups. Also, figure 5 displays the extent of closure of the rabbit pinna affected by the OGC treatment group (Figure 5).

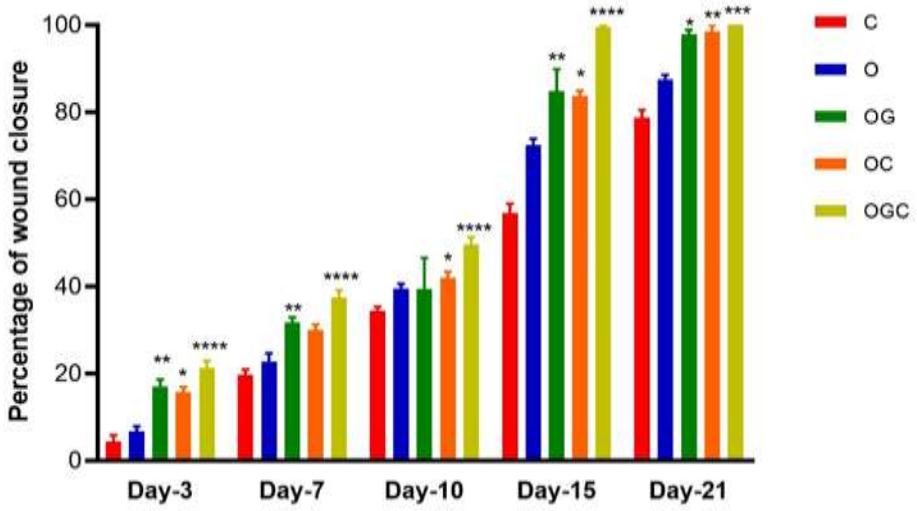

Figure 4. Wound closure; the wound healing process was more pronounced in treatment groups than control groups, C: control group without any treatment, O: groups receiving only the ointment base, OG: the ointment base with bacterial ghost, OC: the ointment base mixed with ECM, OGC: The ointment base mixed with bacterial ghost and ECM. ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ and *$p \leq 0.05$ compared with control group.

Figure 5. Representative pictures of the wound closure on different days on the OGC group.

Microbiology Test

Evaluation of microbial contamination at the site of injury demonstrated that treatments caused a remarkable decrease in bacterial load. The number of colonies in the treatment groups was significantly decreased compared with control groups (Figure 6).

Number of Fibroblast Cells

An increase in the number of fibroblast cells indicates marked acceleration of wound healing. On day 3, the frequency of fibroblast cells was remarkably higher in treatment groups, especially in the OGC group, compared with control groups (Figure 7). On days 7 and 10 post-injury, a significant difference was found in the number of fibroblast cells between treatment and control groups.

Collagen Content

The analysis of collagen content was performed using the measurement of the intensity of blue-colored fibers. As shown in Figure 8, on days 3-10 post-injury, the collagen content was increased in the OGC group compared with other groups, while on day 15, the collagen fibers in the OGC group were comparable with the amounts of collagen fiber seen in normal rabbits (Figure 8). Also, on day 21 post-injury, the collagen content of the OC group was the same as the OGC group.

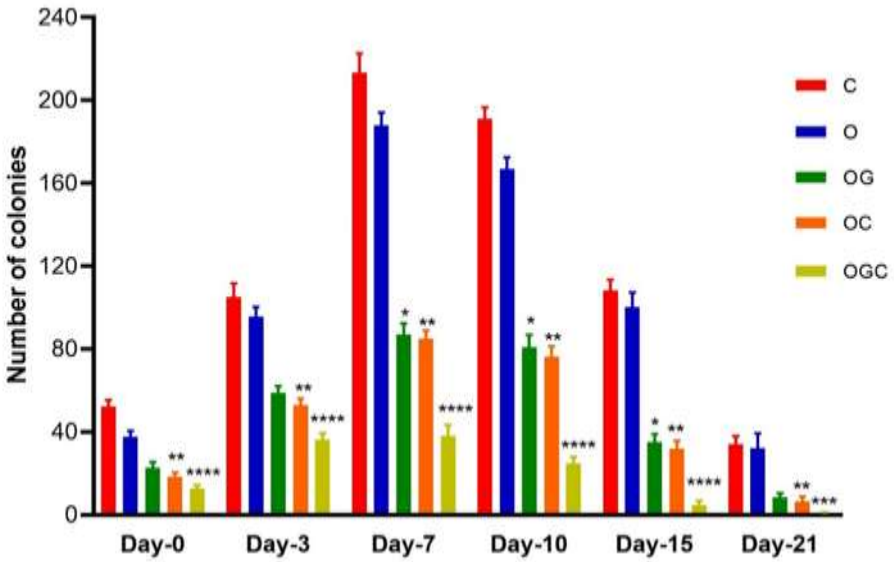

Figure 6. Microbiology of wound healing; on all days, the number of colonies in the treatment groups is decreased compared with control groups. ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ and *$p \leq 0.05$ compared with control group.

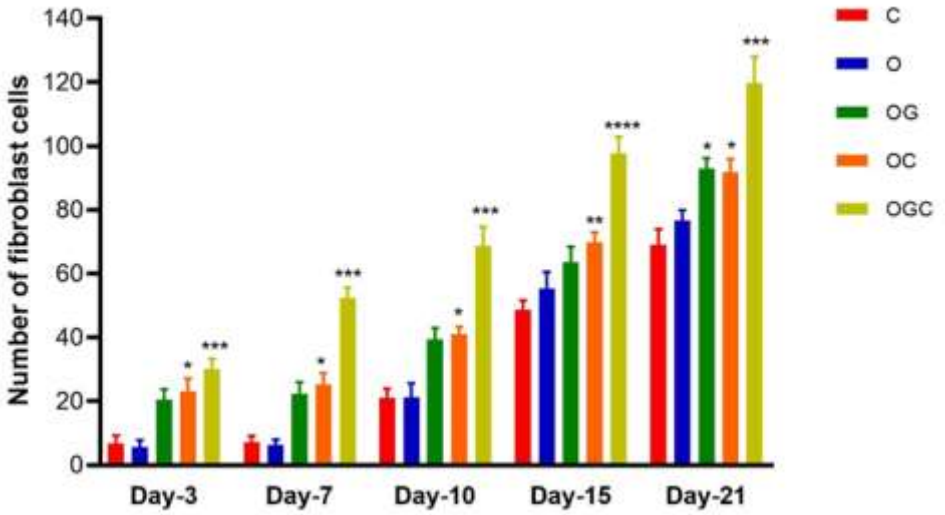

Figure 7. The number of fibroblast cells in control and treatment samples; a significant increase was found in the number of fibroblast cells in treatment groups compared with control groups when the treatment course was ended. ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ and *$p \leq 0.05$ compared with control group.

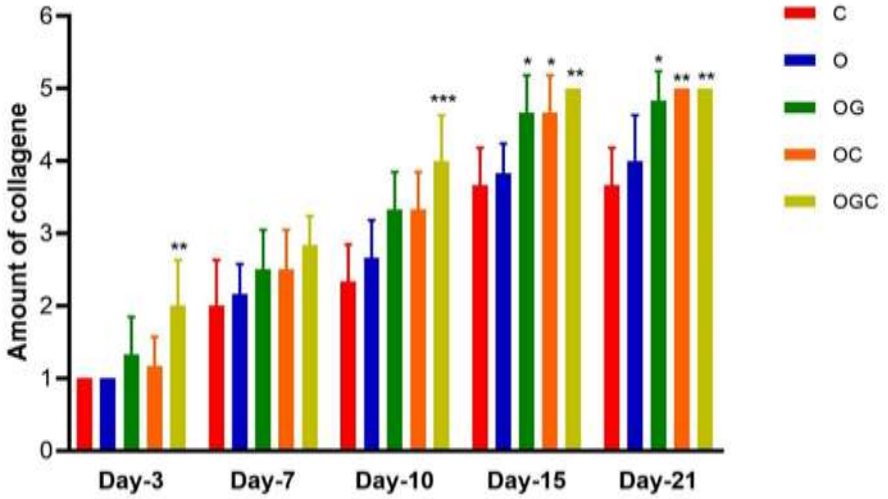

Figure 8. The collagen content; the amount of collagen is substantially higher in treatment groups compared with control groups. ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ and *$p \leq 0.05$ compared with control group.

Discussion

The skin is the first and most crucial defense barrier in mammals; therefore, any damage to this organ must immediately be healed and repaired. Hence, seeking effective treatments with minimum treatment course and side effects would be ideal for the treatment of wounds (Broughton et al. 2006; Velnar et al. 2009). Any rupture in the integrity of skin layers or subcutaneous tissue is called a wound that might be caused by physical or chemical factors. Despite significant advances in wound healing, studies continue to find effective wound healing methods with minimum complications. Therefore, numerous investigations have been conducted on the impact of drugs and wound dressings on accelerating the healing process, such as using calcium, copper, zinc ions, as well as physical factors, such as ultraviolet radiation, electrical stimulation, and laser waves (Reddy et al. 2013; Stanford et al. 1969; Barnett and Varley 1987; Kumar and Jagetia 1995; Brown et al. 1988). The effect of some chemicals, including hydrocortisone, vitamins, phenytoin ointment, saline (Bitar 1997), as well as natural compounds, such as herbs and honey (Al-Waili et al. 2011), was also studied in the literature.

In this study, for the first time, the impact of ECM and BG as novel natural-derived materials on wound healing was assessed. This research aimed to determine whether these natural-derived materials have positive roles in the mitigation of injuries and could be used as a novel biological dressing in the clinic.

Previous studies highlighted the importance of ECM in wound healing (Ghatak et al. 2015). ECM could be obtained from natural tissues. Different types of ECM derived from various tissues have been used as biological scaffolds, such as skin (Brouki Milan et al. 2020), skeletal muscles (Cartmell and Dunn 2000), tendons (Piccoli et al. 2018), small intestinal submucosa (Ji et al. 2019), and liver (Shirakigawa and Ijima 2018). Due to the high amount of GAGs and collagen in the ECM of articular cartilage, bovine articular cartilage was prepared using the decellularization method. Our results have shown that the combination of enzymatic with physicochemical treatments shows a synergistic effect on the complete removal of cellular components as well as the perseveration of ECM contents (Figure 1).

On the other hand, one of the important sources of natural glycoproteins is bacterial cell walls. Bacterial glycoproteins are expressed at the bacterial cell surface and provide natural substances for clinical applications. These compounds play a significant role in interacting with pathogens and identifying their glycan structures (Haidinger et al. 2003). Bacterial ghost contains a variety of surface compounds, such as glycoproteins. The preservation of the natural structure of cell wall components and lack of any genomic or cytoplasmic contents are among exemplary properties of bacterial ghost (Lubitz et al. 2009). In the present study, bacterial ghost was created using the transfection of *E.coli* with the pmET32b plasmid, as previously established at our laboratory (Soleymani et al. 2020). Our findings were confirmed by SEM analysis in which pores in bacterial cell walls were observable (Figure 2, B). After preparing the ECM and bacterial ghost and the mixture of the two compounds with the ointment base (Figure 3), the effect of ECM and bacterial ghost on wound healing of injured rabbit pinna as an experimental model was evaluated at different day intervals.

Wound closure depends on the ability of the type of tissue, animal, and the size of the injury. In the current research, a New Zealand white rabbit was used because the ear pinna of the rabbit wound is an accepted model for the study of wound healing. The pinna tissue is mainly composed of blastema tissue, which is responsible for its capacity in tissue repair (Hashemzadeh et al. 2015).

Due to the microbial load and the initial inflammation on days 3 and 7 post the injury, we did not see a proper wound healing. On the other, our findings showed that the size of the wound was remarkably decreased in the OGC group compared with other groups. On day 15 post-injury, the wound closure was completed in the OGC group, while this process lasted 21 days in the OC group (Figures 4 & 5). Therefore, it seems that the mixture of ECM and bacterial ghost was more effective than other formulations in wound closure. Also, a decrease in bacterial load was inversely correlated with wound closure in treatment groups; in a way, the lowest bacterial load was detected in treatment groups with the highest wound closure (Figure 6).

Fibroblast cells are the most competent cells in wound repair and are considered the main supplier of collagen. The number of fibroblast cells in treatment groups was significantly higher than in control groups (Figure 7). This phenomenon might be due to the presence of PGs and collagen, which possess chemotactic effects on cell polarity and migration. An increase in the number of fibroblast cells led to elevated amounts of collagen in tissues. Collagen is responsible for the tendency of endothelial cells and their physical force to the damaged region. Therefore, as expected, the collagen content was remarkably higher than in the control groups (Figure 8).

Regarding the high amounts of GAGs and collagen in ECM derived from cartilage as well as high amounts of glycoproteins in bacterial ghost, the use of both compounds for wound healing would be beneficial for patients with chronic wounds. Further studies are warranted to delicately unravel the in-vivo effects of biological dressings on wounds in mammals.

Ethics Approval and Consent to Participate

The ethical approval for this study was issued by the Committee on Research Ethics of Ferdowsi University of Mashhad, based on the Ethical Guidelines of Research from the Ministry of Science, Research and Technology of Iran, and following the Declaration of Helsinki.

Consent for Publication

No identifying patient information is included in this report.

Availability of Data and Material

The data used and/or analyzed during the current study are available from the corresponding author on request.

Competing Interests
The authors declare that they have no conflict of interest.

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Authors' contributions
Conceived and designed the experiments: FN, GRH and NMS. Performed the experiments: FN. Analyzed the data: FN, GRH, AT, HN and NMS. Supervised the experiments: GRH and NMS. Wrote the manuscript: FN, GRH and AT. All authors read and approved the final manuscript.

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