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FULL LENGTH PAPER

Preclinical evaluation of decellularized bovine articular cartilage scaffolds for treatment of chronic diabetic wounds in BABL/C mice

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Abstract Chronic diabetic wounds, such as diabetic foot ulcers, pose a significant health challenge due to their prolonged healing times and high recurrence rates. Conventional treatments are often inadequate, driving interest in advanced therapeutic approaches like biological scaffolds. Decellularized scaffolds, which replicate the extracellular matrix (ECM), have shown potential in promoting tissue regeneration and wound healing. This study evaluated the efficacy of decellularized bovine articular cartilage scaffolds in enhancing wound healing in a preclinical murine model of chronic diabetic wounds. Bovine articular cartilage was decellularized using a combination of chemical and physical processes. The scaffolds were characterized through H and E staining (to assess histomorphological characteristics), FTIR, and SEM analyses to confirm ECM preservation and effective decellularization. Twenty female diabetic BALB/c mice were divided into two groups: a control group (treated with Atrauman Ag® dressings) and an experimental group (treated with decellularized bovine articular cartilage scaffolds). This study examined the effects of decellularization on the structural and chemical properties of the cartilage scaffolds, as well as their impact on wound healing and closure rates in diabetic mice compared to the control group. Mice treated with the decellularized cartilage scaffolds demonstrated a significantly faster wound closure rate (100% closure by day 17) compared to the control group (75% closure by day 17, $P < 0.01$). Histological analysis revealed more organized epidermal regeneration, fibrin deposition, and granulation tissue formation in the scaffold-treated group. SEM and FTIR analyses confirmed the preservation and integrity of the ECM before and after the decellularization process. Decellularized bovine articular cartilage scaffolds significantly enhance wound healing in chronic diabetic wounds by promoting tissue regeneration and reducing inflammation. These findings suggest that such scaffolds represent a promising therapeutic option for the treatment of chronic diabetic wounds.

Keywords Decellularized scaffolds · Bovine articular cartilage · Diabetic wounds · Wound healing · Extracellular matrix · Tissue regeneration

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Introduction

Diabetes mellitus is a major global health issue, affecting over 537 million adults worldwide, with a significant proportion residing in low- and middle-income countries. According to the International Diabetes Federation (IDF), the prevalence of diabetes is projected to rise significantly by 2045, particularly in South-East Asia, where cases are expected to increase by 68%, reaching approximately 152 million individuals (Zhang et al. 2017). Among the many complications of diabetes, ischemia, and diabetic neuropathy are particularly severe, often leading to chronic wounds such as diabetic foot ulcers (DFUs). DFUs affect approximately 25% of individuals with diabetes during their lifetime, contributing significantly to both morbidity and healthcare costs (Armstrong et al. 2017). These ulcers are complex, occurring in nearly 60% of diabetic patients with neuropathy due to impaired circulation, loss of sensation, and a dysfunctional inflammatory response (Reiber et al. 1999). Without timely treatment, DFUs can progress to infections, gangrene, and even amputation. The global prevalence of DFUs is estimated to be between 5.5 and 7.3%, with regional variations; for instance, Iran reports rates as high as 15% (Alavi et al. 2014). Chronic DFUs often take over 12 months to heal, with recurrence rates reaching up to 70%, further exacerbating the healthcare burden (Liu et al. 2018). Chronic wounds, including DFUs, disrupt the normal phases of wound healing: hemostasis, inflammation, proliferation, and remodeling. Imbalances in these processes result in non-healing or refractory wounds. Effective treatment strategies aim to reestablish tissue hemostasis, reduce inflammation, stimulate cell proliferation and differentiation, and promote angiogenesis and re-epithelialization of damaged tissue (Liu et al. 2019). Recent advancements in wound care have progressed beyond traditional dressings to incorporate more sophisticated treatments such as gene therapy, platelet-rich plasma (PRP) therapy, and both cell-based and cell-free therapies (Crapo et al. 2011). Among these, biological scaffolds, which mimic the extracellular matrix (ECM) and provide structural support, have shown considerable promise in accelerating tissue regeneration. These scaffolds enhance cellular migration, proliferation, and angiogenesis, all of which are crucial for wound healing. A particularly promising type of scaffold involves the use of mesenchymal stem cells (MSCs) (Wang et al. 2015). MSCs are multipotent stromal cells capable of differentiating into various cell types, such as osteoblasts, chondrocytes, and adipocytes. They exhibit high proliferative capacity, immunomodulatory properties, and paracrine factor secretion, all of which contribute to enhanced wound healing (Gilbert et al. 2006). When incorporated into scaffolds, MSCs promote collagen deposition, reduce inflammation, and encourage the formation of new blood vessels.

Decellularized scaffolds, in particular, provide a biocompatible matrix that retains the ECM's structural components while eliminating immunogenic cellular materials that might otherwise provoke an immune response (Yang et al. 2018). Among the various scaffolds studied, decellularized cartilage has garnered significant attention due to its unique properties. Derived from bovine joint cartilage, these scaffolds offer high mechanical strength and durability, making them ideal for chronic wound environments (Elder et al. 2010). The decellularization process removes cellular components while preserving the ECM, which is crucial for maintaining structural integrity, promoting cellular migration, and supporting angiogenesis and re-epithelialization (Chahal et al. 2013). Articular cartilage, characterized by its elasticity, avascular nature, and unique ECM composition, presents several advantages as a scaffold for wound healing. Its resistance to wear and capacity to withstand mechanical stress make it an excellent candidate for use as a biocompatible dressing for chronic diabetic wounds (Gaharwar et al. 2017). Moreover, decellularized bovine articular cartilage retains its structural integrity while minimizing the risk of adverse immune reactions when applied to wounds (Oryan et al. 2017).

This study aims to evaluate the efficacy of decellularized bovine articular cartilage scaffolds in promoting the healing of chronic diabetic wounds in a preclinical murine model. Bovine articular cartilage was selected for its mechanical strength and ECM composition, which support cellular infiltration and proliferation. By comparing these scaffolds with a commercially available wound dressing (Atrauman Ag®), the study seeks to assess their potential to accelerate wound closure, enhance collagen deposition, and reduce inflammation. Ultimately, this research aims to demonstrate the therapeutic potential of decellularized bovine cartilage scaffolds as

a biocompatible solution for tissue regeneration in chronic diabetic wounds.

Material and method

Preparation and decellularization of bovine articular cartilage

Bovine joints were obtained from a local slaughterhouse in Mashhad. The soft tissues were carefully removed using a scalpel, and articular cartilage was harvested from the femoral condyles shortly after slaughter. The cartilage was sectioned into cylindrical discs with a diameter of 5 mm using a trephine and thoroughly washed with normal saline. The samples were then stored at -4°C for one week prior to the decellularization process. Decellularization was performed using a combination of physical and chemical methods. The frozen samples underwent five cycles of rapid freezing and thawing to facilitate cellular disruption. Following this, the cartilage discs were treated with varying concentrations of sodium dodecyl sulfate (SDS) solutions (1%, 2%, 4%, and 8%) for different durations (1 h, 4 h, and 8 h). After SDS treatment, the samples were thoroughly washed in phosphate-buffered saline (PBS) for 2 h to remove residual detergent. An untreated control group was included for comparative analysis.

Characterization of decellularized articular cartilage scaffold

Histological analysis

The effects of the decellularization process and the preservation of extracellular matrix (ECM) components were evaluated using histological techniques. Normal and decellularized cartilage tissues were fixed in Bouin's solution, dehydrated through a graded ethanol series, embedded in paraffin, and sectioned at a thickness of 7 µm. The sections were stained with Toluidine Blue to confirm the presence or absence of cellular material and to assess the integrity of the ECM.

FTIR analysis of decellularized bovine articular cartilage

Fourier-transform infrared (FTIR) spectroscopy was conducted to analyze the biochemical composition of the decellularized cartilage and compare it with the Atrauman Ag® scaffold as a control. Samples were prepared by freeze-drying and subsequently ground into a fine powder. FTIR spectra were recorded using a Nicolet iS50 FTIR Spectrometer (Thermo Fisher Scientific, USA), with measurements taken in the range of 4000–400 cm^{-1}. Absorption bands, including amide I and amide II, were analyzed to evaluate protein content and identify structural changes.

SEM analysis of decellularized bovine articular cartilage

SEM analysis was used to examine the surface morphology of the decellularized cartilage and the Atrauman Ag® scaffold. For this purpose, samples were fixed in 2.5% glutaraldehyde, dehydrated through a graded ethanol series, and dried using a critical point dryer. Then, they sputter-coated with gold palladium and examined under a JEOL JSM-7500F Field Emission Scanning Electron Microscope. Different images were taken at various magnifications to evaluate surface characteristics and pore structure.

In vivo study

Animals and ethical approval

Approximately, 20 female BABL/C mice (8–12 weeks, 15–20 g) were purchased from the Animal House of Ferdowsi University of Mashhad and housed in the Animal Facility of the Faculty of Basic Sciences, Mashhad. The mice were maintained under controlled environmental conditions, including a temperature range of 22–25 °C, with a 12-h light/dark cycle. All mice were provided with standard chow and water ad libitum. The cages were cleaned daily, and the mice were handled in compliance with ethical guidelines for animal research.

This study, conducted between 2023 and 2024, was a preclinical investigation designed to evaluate the efficacy of decellularized bovine articular cartilage scaffolds in the treatment of chronic diabetic wounds

in BABL/C mice. The research was approved by the Institutional Animal Care and Use Committee of the Motamed Academic Research Institute, following all established guidelines (Approval ID: IR.ACECR.AEC.1400.010). The primary objective of this study was to assess the scaffolds' ability to enhance wound healing through collagen deposition, reduced inflammation, and accelerated tissue regeneration.

Experimental design

At first, the diabetes was induced in mice by a single i.v. Injection of streptozotocin at a dose of 50 mg/kg (STZ, Sigma Chemical Co., St. Louis, MO, USA) dissolved in citrate buffer pH 4.5. Blood glucose concentration was measured to confirm diabetes-induced hyperglycemia at the beginning of the protocol and 24 h after STZ injection (Accu-Check Instant test, Boehringer Mannheim, Germany). Twenty diabetic mice were randomly divided into two groups including the control (n = 10) and experimental (n = 10) groups. The control group received the Atrauman Ag® scaffold (cat. number 4049500271349, Hartmann, Northlands), while the experimental group was treated with decellularized bovine articular cartilage scaffolds.

Wound induction and wound closure assay

A full-thickness skin ulcer was established in BABL/C mice approximately 3 weeks after hyperglycemia (glucose level less than 300 mg/dL) induced by intraperitoneal injection of streptozotocin. For this purpose, diabetic BABL/C mice were anesthetized by intraperitoneal injection of 10 g/L pentobarbital sodium (0.4 mL/100 g). After anesthesia, the back of the mice was shaved and cleaned with povidone-iodine. A circular full-thickness wound of 1 cm in diameter was created on the dorsal skin of the mice using a biopsy punch and the defect area was fixed with a ring-shaped silicone. In the control group, 10 diabetic BABL/C mice received the Atrauman Ag® scaffold (cat. number 4049500271349, Hartmann, Northlands). The other diabetic mice were treated with decellularized bovine articular cartilage scaffolds. The wound dressings in each group changed every other day. The wound healing rate was evaluated at days 0, 7, 12, 17, and 22 post-operations. Wound area and the speed of wound closure detected by image-j software. The wound healing rates were calculated as follows: primary wound size—residual wound size/original wound size × 100%.

For histological analysis, wound areas including the surrounding skin, were collected on days 12 and 22 after operation. For this purpose, the samples were fixed in 4% paraformaldehyde (Solarbio), gradually dehydrated, embedded in paraffin, and cut into 5μm sections. The sections were evaluated with H&E staining, according to the manufacturer's instructions (Sigma-Aldrich). The new fibroblast and inflammatory cells, fibrin deposition, epidermal regeneration, and the formation of granulation tissue were evaluated on days 12 and 22 by two blind pathologists.

Statistical analysis

Data were analyzed using SPSS version 25.0 Statistical significance was determined using t-tests and ANOVA, with a P value of < 0.05 considered significant.

Results

Histopathological findings of bovine articular cartilage scaffolds

Histopathological examination of bovine articular cartilage scaffolds, both pre-and post-decellularization, is presented in Fig. 1A and B. The decellularization process effectively removed all cellular components from the articular cartilage scaffolds, while the extracellular matrix (ECM) was well preserved (Fig. 1B). Toluidine Blue staining confirmed the absence of any cellular material in the decellularized cartilage scaffolds, indicating that the overall structure of the ECM remained intact and undamaged (Fig. 1B).

FTIR and SEM results of decellularized bovine articular cartilage scaffolds

FTIR analysis provided valuable insights into the functional groups present within the decellularized bovine articular cartilage scaffolds and their interactions. This analytical tool allowed the investigation of hydroxyl, amide, and carboxylic acid groups, which are commonly found in the structure of many

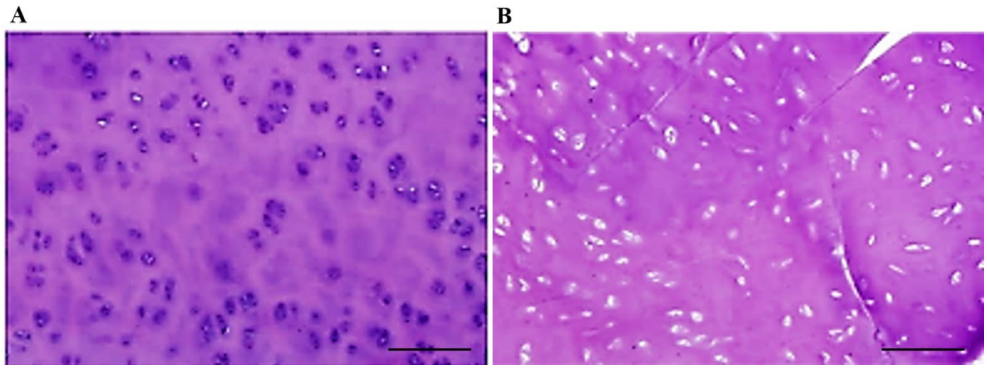


Fig. 1 Histological characteristics of bovine articular cartilage before decellularization (**A**) and after decellularization (**B**) by Toluidine Blue staining, shown at 40×magnification

scaffolds. FTIR analysis in the present study demonstrated that the decellularization process did not adversely affect the biochemical structure of the bovine articular cartilage scaffolds. There was no statistically significant difference in the chemical structure before and after the decellularization process ($P > 0.05$, Fig. 2).

SEM analyses of the bovine articular cartilage scaffolds before (Figs. 3A–C) and after (Fig. 3a–c) decellularization are demonstrated with magnification of 20µm, 10µm, and 5µm. The decellularization process successfully removed all cellular components from the bovine articular cartilage scaffolds while preserving the ECM structure of the cartilage (Fig. 3).

Wound healing process and closure rate

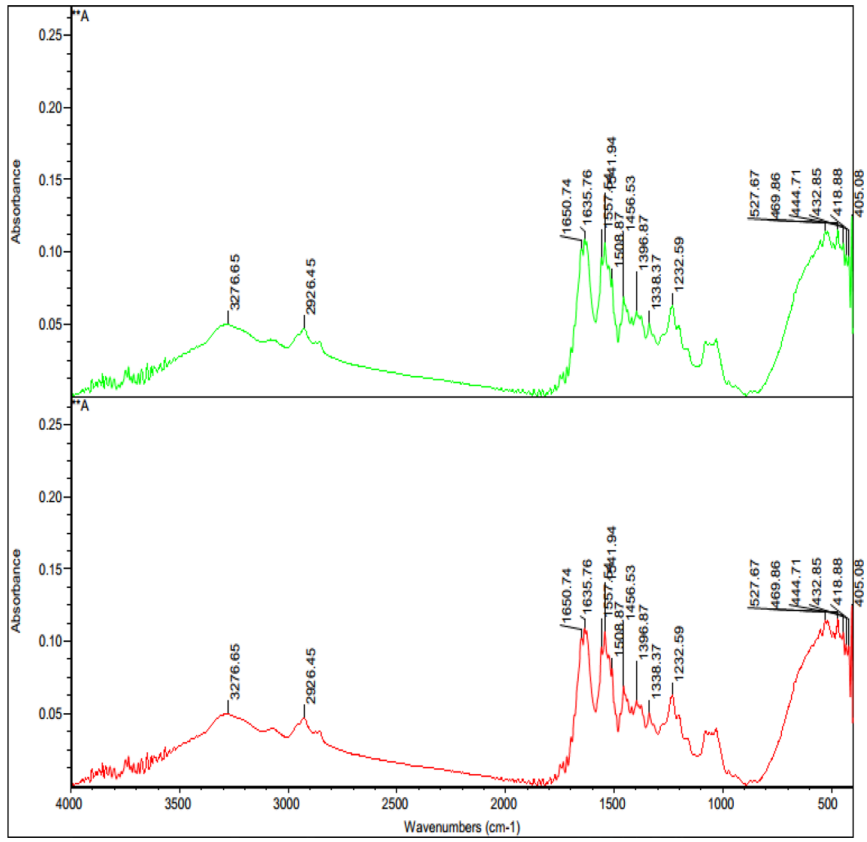
Figure 4 demonstrates the wound healing process in mice with chronic diabetes (Fig. 4A–E) compared to those without diabetes (Fig. 4a–e) at days 0, 7, 12, 17, and 22. In normal mice (without chronic diabetes), the wound healing occurred naturally due to the skin's elasticity, so the wound area decreased by 40%, 60%, 80%, and 100% on days 7, 12, 17, and 22, respectively (Fig. 4A–E). However, in diabetic mice, the wounds do not heal due to high blood sugar (more than 300mg/dl) and subsequent neuropathy (Fig. 4a–e).

The wound healing process in chronic diabetic mice treated with the Atrauman Ag® and decellularized bovine articular cartilage scaffolds is demonstrated in Fig. 5. The right ulcers were treated with decellularized bovine articular cartilage scaffolds, while the left wounds received the Atrauman Ag® scaffolds (Fig. 5). Seven days after treatment with the decellularized bovine articular cartilage scaffolds, a significant reduction (60%) in wound area was detected ($P < 0.05$, Figs. 5), whereas the reduction in wound area in the control group (mice treated with the Atrauman Ag® scaffold) was less pronounced (28%) (Fig. 5B).

On day 17, all treated ulcers with decellularized bovine articular cartilage scaffolds completely healed (100%), compared to only a 75% healing rate in the control group ($P < 0.01$, Figs. 5C). The chronic diabetic ulcers closed after 22 days in both the control (the Atrauman Ag®) and decellularized bovine articular cartilage scaffolds (Fig. 5E). As well as, the wounds in the group treated with decellularized bovine articular cartilage scaffolds have less redness, pain, inflammation, and swelling compared to the Atrauman Ag® group, suggesting faster and more effective wound healing in this group.

Graph analysis of wound healing progress

The graphical representation of wound area reduction over time illustrates distinct differences between the treatment and control groups. On day 7, there was no statistically significant difference in wound size among the groups ($P > 0.05$), as shown by the relatively similar bar heights in the graph. By day 12, although no significant difference was observed in the wound area reduction among the groups, a slight trend toward improvement was noticeable in the treatment groups. On day 17, a significant reduction



1600-1650	Amide I bond, stretching of the carbonyl group (C=O)
1510-1560	Amide II bond, stretching of the C-N group, and bending of the N-H group
1210-1300 and 1070-1080	
1635-1650	Secondary structures of proteins (beta sheets and random coils)

Fig. 2 FTIR analysis of bovine articular cartilage scaffolds before and after decellularization, showing no statistically significant differences in the chemical structure

in wound size was evident in the treatment groups compared to the control ($P \leq 0.05$), as represented by the notable decrease in the respective bars. This trend continued, and by day 22, the treated groups demonstrated a considerable reduction in wound area ($P \leq 0.05$), while the control group showed slower healing progress, as indicated by the higher bar representing a larger remaining wound area.

Overall, the graph visually confirms that the applied treatment played a significant role in accelerating wound healing, with the most pronounced effects observed from day 17 onwards (Fig. 6).

Histopathological characteristics of the wounds after treatment

The histopathological characteristic of healthy skin with the mature stratified squamous epithelium in diabetic mice (before wound induction) is shown in Fig. 7A. The dermis was composed of mature dense collagenous connective tissue and well-organized skin appendage. The epidermis exhibited a typical arrangement of keratinocytes with a clear stratum corneum, indicating proper barrier function (Fig. 7A). Approximately 12 days after treatment,

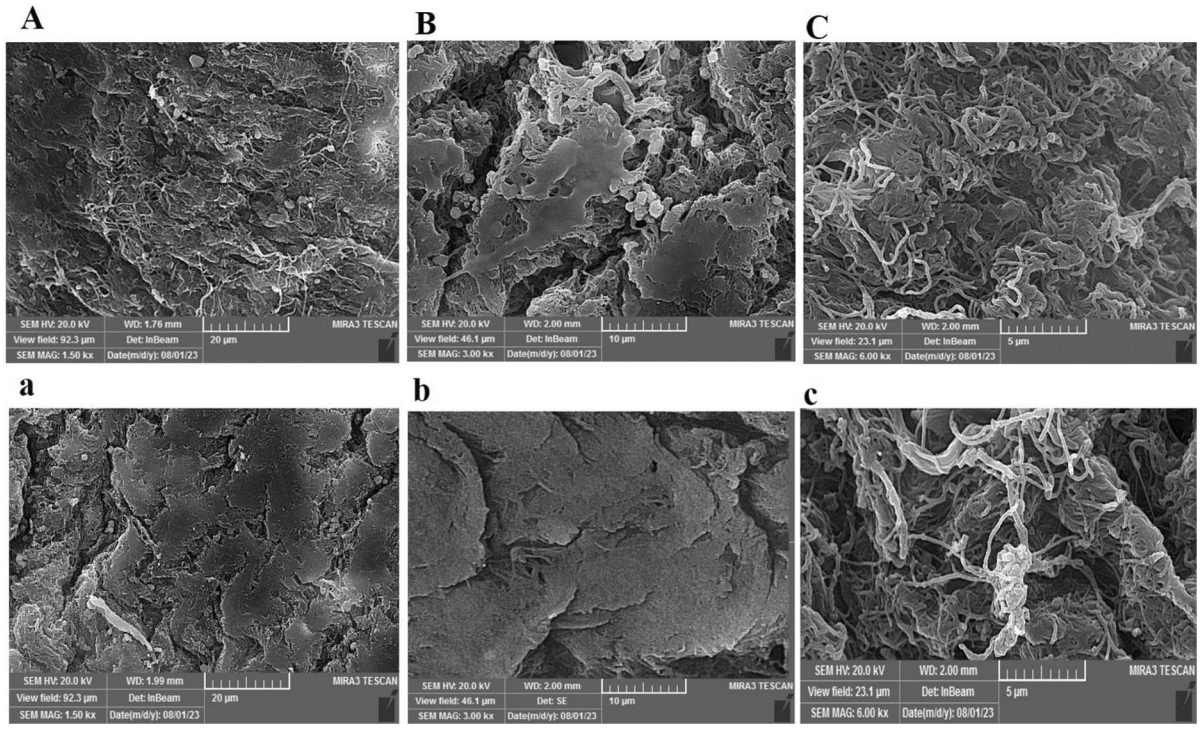


Fig. 3 SEM analyses of the bovine articular cartilage scaffolds before (**A**–**C**) and after decellularization (**a**–**c**), magnification of 20 µm (**A**, **a**), 10µm (**B**, **b**), and 5µm (**c**, c)

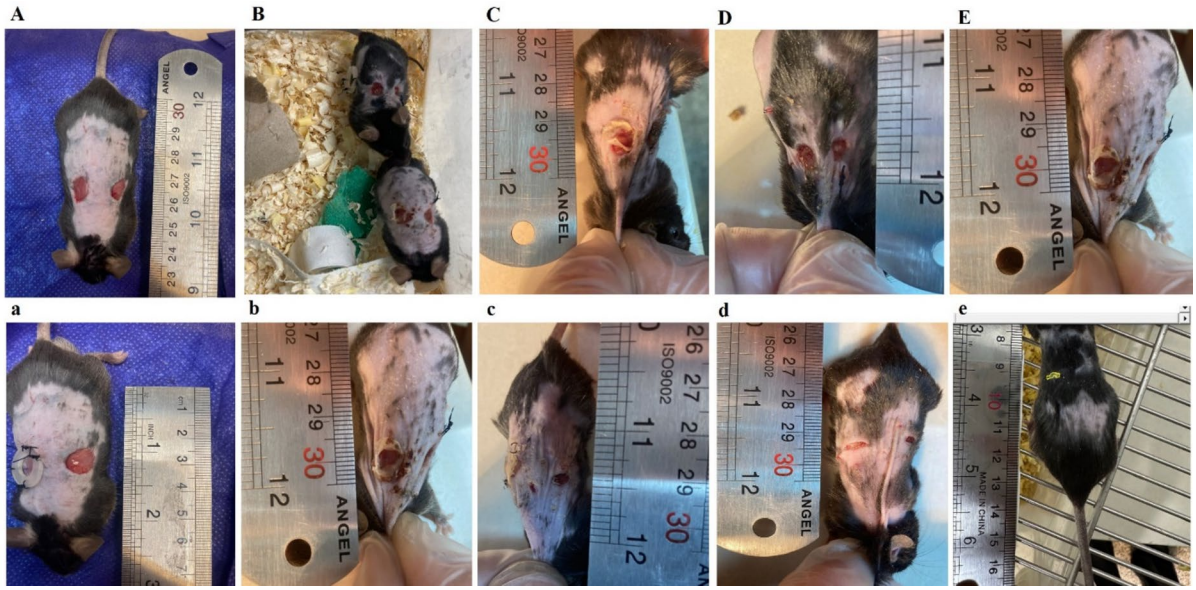


Fig. 4 Wound healing process in mice with chronic diabetic wounds (**A**–**E**) compared to non-diabetic mice (**a**–**e**) at days 0 (**A**, **a**), 7 (**B**, **b**), 12 (**C**, **c**), 17 (**D**, **d**), and 22 (**E**, **e**)

Fig. 5 Wound healing process in the chronic diabetic mice before (**A**), 7 days (**B**), 12 days (**C**), 17 days (**D**), and 22 days (**E**) after treatment with decellularized bovine articular cartilage scaffolds (right ulcers) and the Atrauman Ag® scaffolds (left wounds)

the histological characteristics of wounds received the Atruman® and decellularized bovine articular cartilage scaffolds are shown in Fig. 6B and C, respectively. In the Atruman group after 12 days, we observed the immature granulation tissue and fibrin deposition along with remnant materials (Fig. 7B). Lack of epidermal reformation and immature granulation tissue in the dermis was shown in wounds treated with decellularized bovine articular cartilage scaffolds after 12 days (Fig. 7C). After 22 days, the relatively little mature stratified squamous epithelium was detected in the Atruman ® group. Whereas, the newly formed dermis with the stratified squamous epithelium and mature granulation was demonstrated in the decellularized articular cartilage scaffolds (Fig. 7E and F).

Discussion

The present study evaluated the efficacy of decellularized bovine articular cartilage scaffolds in promoting the healing of chronic diabetic wounds in a murine model (BABL/C mice). Our findings demonstrated

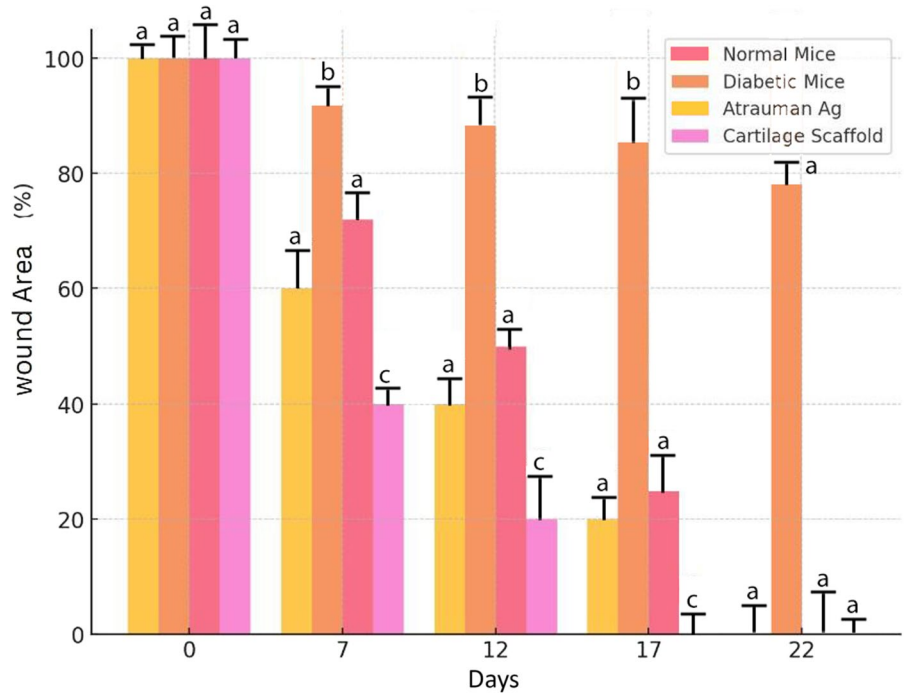


Fig. 6 Wound healing process in different groups of mice

that the scaffold significantly accelerated wound closure, improved collagen deposition, and reduced the inflammatory response compared to conventional wound dressings, specifically the Atrauman Ag® scaffold. These promising results highlight the potential for decellularized bovine articular cartilage to serve as an innovative and effective therapeutic scaffold for chronic diabetic wounds, which are notoriously difficult to heal.

The decellularization process used in this study successfully preserved the extracellular matrix (ECM) of the bovine articular cartilage while eliminating immunogenic cellular components. The ECM's retention of structural proteins such as collagen, as confirmed by FTIR and histological analysis, provided a biocompatible environment conducive to cell attachment, proliferation, and differentiation—key elements of effective wound healing. The scaffold's porous structure facilitated fibroblast infiltration, which is crucial for collagen synthesis and tissue remodeling. These properties likely contributed to the enhanced wound healing observed in the scaffold-treated group, particularly in terms of faster re-epithelialization and more organized collagen deposition.

Moreover, the scaffold-treated wounds exhibited significantly lower levels of inflammation compared to the control group. The reduction in redness, swelling, and overall inflammatory cell infiltration suggests that the decellularized scaffold may have immunomodulatory properties, helping wounds transition more quickly from the inflammatory phase to the proliferative and remodeling phases. This characteristic is particularly important in chronic diabetic wounds, which often remain in a prolonged state of inflammation that delays healing. These results are consistent with previous studies on decellularized scaffolds, which have shown that preserved ECM can modulate immune responses and promote tissue repair (Oryan et al. 2017; Chen et al. 2010). The results of this study align with a growing body of research demonstrating the therapeutic potential of decellularized scaffolds for chronic wound healing. Similar to the findings of (Liu et al. 2007), who reported that decellularized bovine pericardium scaffolds promoted cellular infiltration and angiogenesis in chronic wound models, the bovine articular cartilage scaffold used in this study significantly enhanced fibroblast activity and collagen organization. Additionally, our results reflect those of (Oryan et al. 2018), who found that decellularized scaffolds derived from other tissues (e.g., trachea) supported rapid tissue regeneration through preserved structural integrity and immunomodulatory

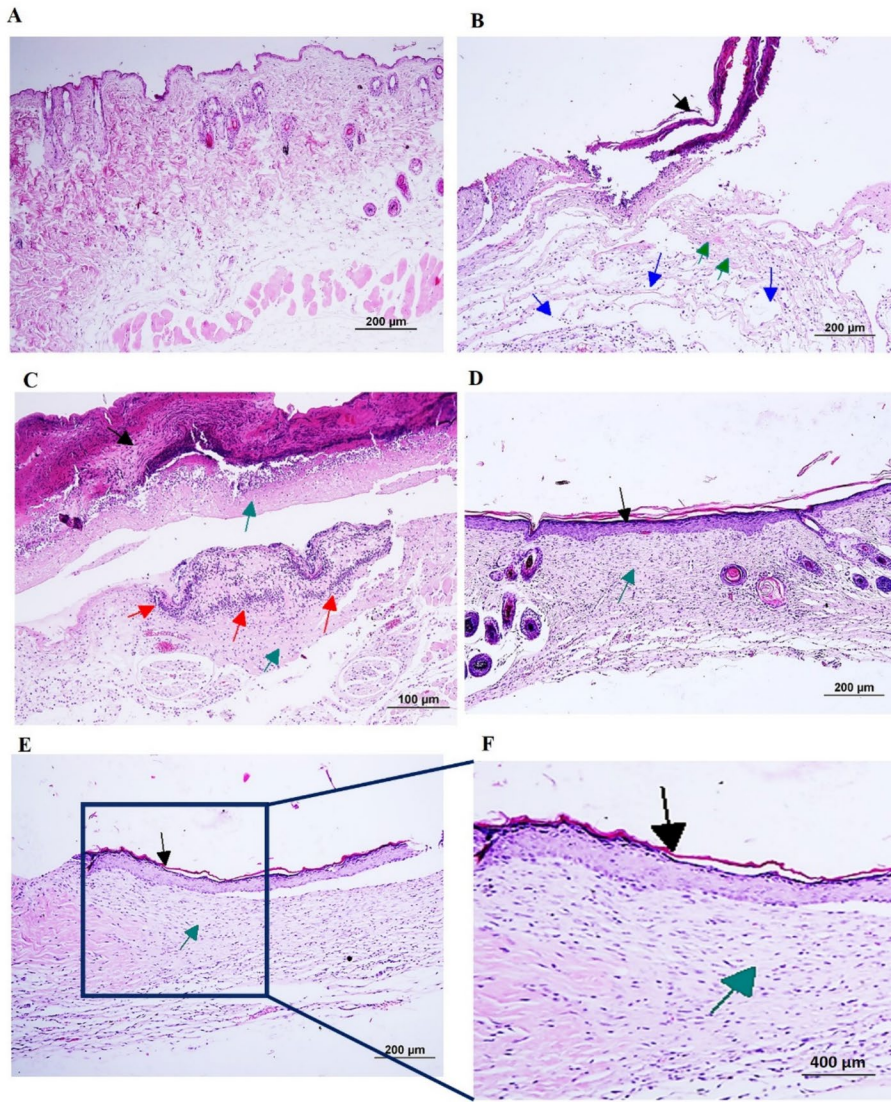


Fig. 7 Normal skin before ulceration (**A**). Histological characteristics of wounds treated with the Atruman ® (B) and decellularized bovine articular cartilage scaffolds (**C**) after 12 days. Note the lack of regeneration of the epidermis, fibrin deposition with residual material (green arrow), immature granulation tissue (blue arrow), and crust (black arrow) of the dermis wound surface in the Atruman ® group after 12 days (**B**). At the same time, 12 days after treatment with decellularized articular cartilage scaffolds, note a lack of epidermal reformation and immature granulation tissue in the dermis. Scab (black arrow), the remnant of scaffold and fibrin deposit (green arrow), and also immature dermis with noticeable infiltration of acute inflammatory cells (red arrow) were detected (**C**). After 22 days post-treatment, in the Atruman ® group, note the relatively little mature stratified squamous epithelium (black arrow) (**D**). In the decellularized articular cartilage scaffolds group, note stratified squamous epithelium (black arrow) and mature granulation tissue in the newly formed dermis (green arrow) (**E**, **F**)

effects. However, unlike softer tissue-derived scaffolds, such as amniotic membrane scaffolds studied by (Osman et al. 2020), the decellularized bovine articular cartilage scaffold in our study offers a higher degree of mechanical stability. This feature is particularly advantageous in treating chronic diabetic wounds, where external stress and mechanical strain can complicate healing. The superior mechanical strength of articular cartilage provides a stable framework for cellular activity while withstanding

the physical demands of the wound environment, which may explain the observed improvements in wound healing outcomes. Furthermore, the decellularized bovine articular cartilage scaffold in this study performed better in reducing inflammation compared to scaffolds derived from other tissues, such as those explored by (Naasani et al. 2016). In their study on burn wounds, they reported slower epithelialization when using decellularized scaffolds without additional bioactive agents. In contrast, the results of our study suggest that the decellularized articular cartilage scaffold alone is sufficient to accelerate epithelialization, though future studies could explore combining the scaffold with growth factors or stem cells to further enhance regenerative potential. While the findings from this study are encouraging, several limitations must be acknowledged. The use of a murine model, though suitable for preclinical evaluation, may not fully replicate the complexity of diabetic wounds in human patients. Larger animal models or human clinical trials will be necessary to confirm the scaffold's efficacy in more clinically relevant settings. Additionally, the long-term effects of the scaffold on scar formation and tissue functionality were not assessed in this study, and future research should explore these outcomes to better understand the potential for scar-free healing and functional tissue regeneration. Another limitation is that this study did not explore the use of the decellularized bovine articular cartilage scaffold in combination with bioactive agents or mesenchymal stem cells (MSCs). Prior research has shown that scaffolds seeded with MSCs or loaded with growth factors can further enhance tissue regeneration by promoting angiogenesis and reducing fibrosis (Fong et al. 2011). Exploring these combinations in future studies could optimize the scaffold's therapeutic potential.

Conclusion

In summary, the obtained data indicate that the use of decellularized bovine articular cartilage scaffolds significantly accelerates the healing process of diabetic wounds and improves the quality of tissue repair compared to the control group ($P < 0.01$). These scaffolds facilitate faster and more effective wound healing by providing a conducive environment for cell growth and proliferation, reducing inflammation, and enhancing collagen fiber formation. The results suggest that these scaffolds could be considered an effective and sustainable therapeutic option for treating chronic diabetic wounds in preclinical models.

Author contribution Nazanin Akbari: Conceptualization, Methodology, Investigation, Data Curation, Writing—Original Draft. Shaghayegh Tafazoli: Formal Analysis, Supervision, Writing—Review and Editing. Banafsheh Heidari: Project Administration, Funding Acquisition, Supervision, Writing—Review and Editing.

Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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