

The application of Mesenchymal Stem Cell-derived Exosomes Implanted on ovine small intestine submucosa in healing of Full-Thickness Cutaneous Wound in male rats

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Abstract

Introduction Treatment of full-thickness injuries poses challenges since these injuries tend to be hard to treat and come with high chances of infection, scarring, and other health risks. Traditional treatment methods such as skin grafting have several limitations, while current substitutes are unable to replace normal skin completely. Treatment using stem cell-derived exosomes has been proven efficient, although there are concerns regarding retention of exosomes in the area of injury. The use of exosomes combined with a scaffold material such as SIS can improve treatment efficiency by providing physical support and sustained therapy.

Materials and Methods Decellularized SIS scaffolds were obtained from a bovine source and characterized through DAPI staining and DNA measurement. MSC exosomes from human umbilical cords were isolated via ultracentrifugation and characterized through dynamic light scattering, transmission electron microscopy, flow cytometry, and Western blotting. Full-thickness wounds were inflicted on rats and treated with either SIS or SIS-Exo scaffold, and left untreated wounds served as controls. The wound healing was analyzed on day 14 by performing H&E and Masson's trichrome staining along with semi-quantitative histological scoring and ImageJ analysis.

Results SIS-Exo treatment showed significant improvement in wound healing in comparison to SIS and untreated controls. Histological evaluation revealed better granulation tissue formation, re-epithelialization, angiogenesis, and deposition of collagen in SIS-Exo treatment. Masson's trichrome staining revealed a significant increase in collagen deposition in SIS and SIS-Exo treatment as compared to the control, but higher collagen deposition was seen in the SIS-Exo group ($P < 0.001$).

Conclusions The combination of SIS with exosomes (SIS-Exo) has proven to be highly effective in wound healing in rats, improving tissue regeneration, collagen formation, and vasculature formation. There is a great deal of promise in its application in regenerative medicine; however, more studies are required to prove its efficacy.

Introduction

Full-thickness wounds, which are described as those involving damage extending to the entire dermis layer of the skin, may pose significant challenges due to the poor regenerative abilities of these wounds and their complications [1, 2]. Full-thickness wounds can occur due to traumas, burns, and other underlying conditions and are characterized by slow wound healing, risk of infections, increased inflammation, as well as possible formation of scars or loss of function [3]. The treatment of chronic wounds, which include diabetes ulcers, venous ulcers, pressure sores, and burns, represents an important health issue, with over 7 million affected Americans every year, resulting in annual spending above \$25 billion [4, 5]. In particular, the treatment of a single diabetic foot ulcer alone may be estimated at \$50,000 [6]. Full-thickness skin wounds also play an important role in morbidity and mortality rates, with nearly 500,000 burn victims seeking medical help annually in the United States [5].

Autologous split-thickness skin grafting remains the standard treatment for full-thickness skin defects and deep burns [7]. However, this approach is often associated with limitations, including donor site morbidity, severe pain, hypertrophic scarring, and suboptimal cosmetic and functional outcomes [8]. In situations of severe skin injury, temporary solutions such as wound dressings, cadaveric skin, or

xenogeneic grafts are commonly used to reduce fluid loss and prevent infection [9, 10]. Despite these measures, wounds may remain open for prolonged periods, increasing the risk of complications [11]. Consequently, artificial and biological skin substitutes have been developed to enhance healing; however, achieving complete and functional regeneration of full-thickness skin remains challenging [12, 13]. Currently available tissue-engineered constructs do not fully replicate the structural and functional properties of native skin nor consistently outperform autologous grafts [14], although dermal substitutes are widely utilized in reconstructive procedures. In this context, emerging strategies such as small intestinal submucosa (SIS) and Stem cell-based therapies have shown promise as potential alternatives to improve wound healing outcomes [13, 15, 16].

Stem cell-based therapies have emerged as a promising strategy for enhancing full-thickness wound healing due to their ability to promote tissue regeneration, angiogenesis, and extracellular matrix production [17]. However, their clinical application remains limited by safety concerns such as uncontrolled differentiation, tumorigenicity, immunogenicity, and challenges related to cost, handling, and delivery [18, 19]. Increasing evidence indicates that the therapeutic effects of stem cells are largely mediated by their extracellular secretions, particularly exosomes, nanosized (30–150 nm) lipid bilayer vesicles enriched with proteins, lipids, and nucleic acids that regulate key cellular processes involved in wound repair [20]. Mesenchymal stem cell (MSC)-derived exosomes, especially from adipose and placental sources, have shown significant potential in full-thickness wound healing by enhancing cell migration, proliferation, angiogenesis, collagen synthesis, and re-epithelialization [21]. Compared to cell-based therapies, exosomes offer advantages including lower immunogenicity, improved stability, easier handling, and greater reproducibility. Nevertheless, their rapid clearance, short half-life, and limited retention at the wound site reduce their therapeutic efficacy [22, 23].

To address these limitations, tissue engineering approaches have focused on integrating exosomes into biomaterial-based scaffolds that enhance their stability and enable sustained, localized release [24]. An ideal scaffold should mimic the extracellular matrix, support cell adhesion and proliferation, and provide a moist, protective environment for healing [25]. Collagen-based systems, designed to mimic the natural extracellular matrix of the skin, have shown promising results in promoting cell proliferation, migration, and angiogenesis, thereby accelerating wound healing [26]. In this context, SIS, a porcine-derived, acellular, collagen-based matrix that has been tested without seeded smooth muscle cells (SMCs), has attracted considerable attention due to their biocompatibility, structural similarity to native tissue, and ability to facilitate tissue remodeling, and has shown promising results in improving inflammatory injuries such as bone regeneration and perianal fistula [27–29]. Therefore, combining SIS-based scaffolds with exosome delivery represents a promising and advanced strategy for improving outcomes in full-thickness wound healing. In line with these advancements, the current *in vivo* study aims to explore the possibility of using SIS, exosomes, and SIS-Exo as a treatment, compared to a control. The use of both the structural qualities of SIS as a scaffold and the biological activities of exosomes will be assessed to determine the benefits of their combined use in wound healing.

Materials and Methods

Scaffold Preparation

The SIS was kept in a medium consisting of 50 mL of 70% ethanol (Merck) and 50 mL of chloroform (Merck, Germany) with constant stirring for 12 hours. Then, the tissue was washed with normal saline solution, and 0.05% trypsin (Thermo Fisher Scientific Corporation) was added to it, which was left at 37°C for 12 hours. After that, the tissue was washed three times with PBS. Then, 0.05% sodium dodecyl sulfate (SDS; Thermo Scientific™) was added to the tissue and stirred for 6 hours. Finally, the tissue was washed three times with normal saline for 45 minutes each time [30, 31].

Characterization of SIS

The SIS samples were fixed in 10% neutral buffered formalin solution, embedded in paraffin, sectioned, and stained with DAPI to view nuclei of cells. Three samples were studied for each set of experiments. DNA content analysis included the treatment of 100 mg of ground SIS with proteinase K digestion buffer that contained 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% SDS, and 0.1 mg/mL proteinase K at 50°C for 24 hours. Next, DNA extraction from the sample was performed two times with phenol-chloroform-isoamyl alcohol (25:24:1 v/v/v). The aqueous phase was then mixed with two volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2). Then DNA precipitation was carried out, and the mixture was stored overnight at -20°C. Next, DNA was centrifuged at 10,000 × g for 10 minutes and dissolved in 1 mL of TE buffer (pH 8.0), containing 1 mM EDTA [32].

Isolation and Characterization of Human Umbilical Cord-Derived MSCs

Umbilical cord tissues from humans were harvested upon approval from the Institutional Research Ethics Committee (ethical approval ID: IR.TUMS.IKHC.REC.1400.184) based on the research protocols of Imam Khomeini Hospital. After harvesting, umbilical cord tissue specimens were kept in phosphate buffer saline (PBS) containing 100 U/mL Pen/Strep (Gibco).

Blood vessels were dissected out, and the rest of the tissue was mechanically chopped into fragments ranging between 2–6 mm³. Tissue fragments were washed several times with PBS and digested with 2 mg/mL type IV collagenase in a water bath at 37°C for 2 hours. Digested tissue cells were then washed with PBS and filtered through a 70-µm nylon mesh to remove undigested tissue particles and cellular clumps.

The isolated cells were cultured in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and Pen/Strep. Cells were maintained at 37°C in a humidified incubator containing 5% CO₂ and approximately 90% humidity. MSCs obtained from the second and third passages were used for exosome isolation. The multilineage differentiation potential of

MSCs was confirmed by evaluating their ability to undergo adipogenic and osteogenic differentiation according to previously established protocols.

Isolation and Characterization of MSC-Derived Exosomes

The production of exosomes was done through culturing of the third-passage MSCs in T175 culture flasks, using DMEM/F12 media with Pen/Strep and 10% exosome-free fetal bovine serum. The cells were incubated for 48 hours in 37°C incubator containing 5% CO₂. The collected CM was subjected to centrifugations for removing unnecessary cell components. First, centrifugation was done at 400 × g for 10 minutes for removal of the whole cells. After that, the samples were again centrifuged at 2500 × g for 30 minutes to isolate the cellular debris and apoptotic bodies. Finally, the supernatant obtained after the previous steps was ultracentrifuged at 100,000 × g for 120 minutes using a Beckman ultracentrifuge (USA). Again, the supernatant was removed from the solution, and the isolated pellets were ultracentrifuged under the same condition.

The total protein concentration of the isolated extracellular vesicles was quantified using the Bradford colorimetric assay to verify exosome yield. The size distribution and particle characteristics of the isolated exosomes were evaluated using dynamic light scattering (DLS). Expression of typical exosomal surface markers, including CD9, CD63, and CD81, was confirmed by flow cytometry and Western blot analysis. Fluorescein isothiocyanate (FITC)-conjugated anti-CD63 and phycoerythrin (PE)-conjugated anti-CD81 antibodies (BD Pharmingen) were used for flow cytometric detection.

The morphology and size of the isolated exosomes were further examined using transmission electron microscopy (TEM) according to previously reported procedures. Based on previous optimization studies, the effective concentration and volume of exosome administration were determined as 5 mL of exosome suspension at a concentration of 50 µg/mL.

Animal Model, Surgical Procedure, and Ethics

The wound-healing efficacy of the scaffolds was evaluated using a murine full-thickness excisional wound model. All animal procedures were approved by the Animal Ethics Committee of Tehran University of Medical Sciences (Ethical Approval No. IR.TUMS.AEC.1402.039) and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996).

A total of eight healthy rats were included in this experimental study, weighing between 200 to 250 grams and aged 4–6 weeks, obtained from the Experimental Medicine Research Center. Animals were housed under standard laboratory conditions with controlled temperature (22 ± 2°C), relative humidity (50–60%), and a 12-hour light/dark cycle, with free access to standard rodent chow and water throughout the study. Animals were acclimatized for one week before surgery.

General anesthesia was induced by intramuscular injection of ketamine (90 mg/kg) and xylazine (12 mg/kg). Ketorolac was administered as perioperative analgesia. After adequate anesthesia was achieved, the dorsal hair was shaved, and the surgical site was disinfected with povidone-iodine followed by 70% ethanol under sterile conditions.

Each mouse received two circular full-thickness excisional wounds measuring approximately 2 × 2 cm on the dorsal skin. Sterilized scaffolds were trimmed to the desired size and secured to the wound bed using four interrupted 4 – 0 silk and nylon sutures placed at the 12, 3, 6, and 9 o'clock positions. Dressings and treatments were applied according to the assigned intervention protocol.

Animals were monitored daily for signs of infection, wound contraction, and general health status. Standardized digital photographs of the wounds were obtained during the experimental period, and wound closure was quantified using ImageJ software (Version 1.53k, National Institutes of Health, USA).

One animal died during the experimental period for reasons unrelated to the study. Consequently, seven animals completed the experiment and were included in the final analyses. Since each mouse received two dorsal wounds, a total of **14 wounds** were available for histological evaluation. Among these, three wounds were treated with SIS and compared with three corresponding control wounds, whereas four wounds were treated with SIS loaded with exosomes (SIS-Exo) and compared with four corresponding control wounds.

At the predetermined endpoint (day 14), animals were euthanized by carbon dioxide (CO₂) inhalation in accordance with institutional ethical guidelines. Full-thickness skin specimens, including the wound bed and surrounding healthy tissue, were harvested for histopathological evaluation.

Scaffold transplantation

Scaffolds were cut into desirable sizes and sterilized using O3 plasma (low-temperature Hydrogen Peroxide Plasma Sterilizer, Standard Sterilization Time: 45min, Sterilization temperature: 50 ± 5°C, Sterilizing Agent: 60% H₂O₂, MEDFUTURE China). The scaffolds were fixed at the wound sites using four simple sutures on 0, 3, 6, and 12 o'clock borders by 4 – 0 silk and 4 – 0 nylon sutures (Ethicon, Norderstedt, Germany).

Histopathological Evaluation

Excised tissues were fixed in 10% neutral buffered formalin, dehydrated through graded ethanol solutions, cleared in xylene, embedded in paraffin, and sectioned at approximately 4–5 µm thickness.

Hematoxylin and Eosin (H&E) Staining

Histological evaluation was performed by a single blinded pathologist. Sections were deparaffinized at 70°C for 20 minutes and immersed in xylene for 3 minutes. Rehydration was performed using two changes of absolute ethanol for 5 minutes each. Slides were stained with hematoxylin for 15 minutes, rinsed under running tap water for 5 minutes, differentiated in 0.2% ammonia water, and washed again. Counterstaining was performed using eosin-phloxine for 1 minute, followed by dehydration in 90%, 96%, and 100% ethanol for 2 minutes each. Sections were cleared in xylene, mounted, and examined under a light microscope.

Histological assessment included inflammatory cell infiltration, granulation tissue formation and maturation, collagen deposition and maturation, re-epithelialization, and neovascularization. Each parameter was scored using a semiquantitative scale ranging from 0 to 3, with higher scores indicating greater histological response.

Blood vessel density was determined by counting mature blood vessels containing red blood cells in four randomly selected microscopic fields (10× magnification), and the average vessel count was calculated.

Outcome Measures

The outcome was histological wound healing assessed by: collagen deposition, granulation tissue formation, epidermal regeneration, and angiogenesis.

Histopathological analysis Methods

The H&E staining protocol was employed for histopathological evaluation, performed by a single blinded pathologist. Samples were deparaffinized at 70°C for 20 minutes, then immersed in xylene for 3 minutes. Rehydration was carried out with two 5-minute changes of absolute ethanol. Nuclei were stained by immersing the specimens in hematoxylin solution for 15 minutes, followed by washing under tap water for 5 minutes. Slides were then treated with 0.2% ammonia water and rinsed again with tap water for 5 minutes. Counterstaining with eosinophloxine was conducted for 1 minute, and the samples were sequentially rinsed in 90%, 96%, and 100% ethanol for 2 minutes each. The samples were then cleared with xylene, a mounting medium was applied, and coverslips were placed. An optical microscope (10× magnification) was used to evaluate skin tissue regeneration. Blood vessel density was measured by counting the number of mature vessels containing red blood cells in four random fields (10× magnification), and the mean vessel count was calculated.

Masson's Trichrome Staining

Masson's trichrome staining was performed to evaluate collagen deposition and maturation. Following deparaffinization and rehydration, tissue sections were stained sequentially with Weigert's iron hematoxylin, Biebrich Scarlet-Acid Fuchsin, phosphomolybdic/phosphotungstic acid, and aniline blue according to the manufacturer's protocol. Slides were dehydrated, cleared in xylene, mounted, and examined microscopically.

Quantitative assessment of collagen-positive areas and immunohistochemical staining was performed using ImageJ software (Fiji version). The percentage of positively stained area was calculated from representative microscopic fields.

Statistical Analysis Methods

Data were analyzed using IBM® SPSS® 26. One-way ANOVA was used to assess statistical differences, followed by Tukey's post hoc test. Results were expressed as means $\pm$ standard deviation (SD), with a significance level set at P-value < 0.05.

Results

H&E and Trichrome results

On day 14, histological analysis revealed fibroblast infiltration and the initiation of granulation tissue formation in all study groups (Fig. 1. a). Notably, the SIS-Exo exhibited the greatest granulation tissue thickness. Additionally, epidermal regeneration was most pronounced in the SIS-Exo group. However, skin appendages were absent across all intervention groups during histological assessments. Additionally, these findings indicate that the SIS-Exo group demonstrated superior granulation tissue formation, epidermal regeneration, and angiogenesis compared to other groups.

Collagen Deposition (Masson's Trichrome)

One-way ANOVA of MT staining showed significant differences between intervention and control groups (P-value $\leq$ 0.001). Furthermore, Tukey's post hoc test revealed that collagen deposition in the SIS and SIS-Exo groups was significantly higher than in the control group; likewise, significant differences were detected among the intervention groups (Fig. 1. b).

Discussions

The findings of this study demonstrate that the application of SIS-Exo significantly enhanced wound healing in a murine model. Compared to control groups, SIS-Exo treatment resulted in improved granulation tissue formation, increased collagen deposition, and more advanced epidermal regeneration. Histopathological analyses confirmed that, although all groups exhibited initial stages of healing, the SIS-Exo group showed superior tissue organization and regenerative capacity.

Exosome–SIS Synergistic Effects

Our findings demonstrated that the application of SIS-Exo significantly improved wound healing in a murine model. The present findings can be explained by the complementary and potentially synergistic effects of exosomes and SIS in promoting full-thickness wound healing [33]. Exosomes are known to regulate key regenerative processes, including fibroblast proliferation, angiogenesis, collagen synthesis, and re-epithelialization, mainly through the transfer of bioactive molecules such as miRNAs and proteins

that activate signaling pathways (e.g., AKT, ERK, and Wnt/ β -catenin) [34, 35]. In particular, studies have shown that exosomes can enhance macrophage polarization from the pro-inflammatory M1 phenotype to the regenerative M2 phenotype, thereby reducing inflammation and accelerating tissue repair [36, 37]. However, their therapeutic efficacy is often limited by rapid clearance, short half-life, and poor retention at the wound site [38]. In contrast, SIS, as an extracellular matrix-derived scaffold, provides a 3-dimensional microenvironment that supports cell adhesion, migration, and tissue remodeling while maintaining high biocompatibility [39].

Several studies have demonstrated that combining exosomes with different types of scaffolds significantly enhances their therapeutic efficacy in wound healing by improving retention, stability, and sustained release at the injury site [25, 40–42]. Polymer-based scaffolds, particularly injectable hydrogels composed of materials such as gelatin, hyaluronic acid, chitosan, and collagen, have shown favorable structural properties including sizes (≈ 30 – $250\ \mu\text{m}$), high swelling capacity (up to 1670%), and controlled degradation profiles, which collectively support cell infiltration and tissue regeneration [43–45]. These scaffolds consistently exhibited good biocompatibility, along with enhanced cell adhesion, proliferation, and migration in vitro [45]. For example, collagen- and glycosaminoglycan-based hydrogels and composite systems such as gellan collagen or hyaluronic acid–chitosan scaffolds were shown to promote fibroblast activity and wound repair processes [46]. In parallel, biological scaffolds such as decellularized matrices, including SIS, have demonstrated excellent biocompatibility and cell adhesion due to their preserved extracellular matrix architecture [47]. Notably, SIS derived from porcine tissue supports cell attachment and tissue remodeling through its natural composition of collagen and growth factors.

Meta-analysis data further confirm that scaffold-based delivery systems significantly enhance regenerative outcomes when combined with bioactive agents such as stem cells or exosomes, improving wound closure (SMD = 3.97 short-term, 3.47 mid-term, 3.03 long-term), angiogenesis (SMD = 6.24), collagen deposition (SMD = 4.97), and growth factor expression (SMD = 6.68), while reducing inflammation (SMD = – 4.88) [48]. In this context, the results of our study are consistent with previous evidence, as the SIS-Exo group demonstrated superior healing outcomes compared to SIS or exosomes alone. This can be attributed to the capability of SIS to function as a natural extracellular matrix scaffold that enhances exosome retention and controlled release, localized delivery [49]. This synergy likely explains the superior outcomes observed in our SIS-Exo group in our in vivo model compared to SIS, exosome, or control groups alone, thereby maximizing their biological activity and promoting more effective full-thickness wound regeneration.

Similarly, studies investigating exosome-based therapies reported significant improvements in wound healing parameters, with one meta-analysis demonstrating enhanced wound closure (SMD = 2.89, $P < 0.00001$), angiogenesis (SMD = 5.46), collagen deposition (SMD = 3.65), and re-epithelialization (SMD = 5.03), along with reduced scar formation (SMD = – 5.05 [50]). Additional systematic reviews further confirmed that exosome therapies promote collagen and elastin synthesis, improve skin regeneration, and modulate inflammatory responses, highlighting their strong therapeutic potential in wound healing

[21, 51]. In line with the importance of optimized wound environments, one study using a porcine full-thickness wound model demonstrated that a wet environment significantly accelerated healing compared to moist and dry conditions, with 86% re-epithelialization in saline-treated wounds versus 20% and 0%, respectively ($p = 0.0046$ and 0.027), and faster wound contraction ($p < 0.005$) [52].

Role of Hydrogels and Collagen-Based Scaffolds in Wound Healing

Biomaterial-based scaffolds, particularly hydrogels and collagen-based matrices, play a critical role in improving wound healing outcomes by mimicking the natural extracellular matrix and providing a supportive environment for tissue regeneration [53, 54]. Hydrogels are highly hydrated, biocompatible polymers that can maintain a moist wound environment, facilitate gas exchange, reduce pain, and serve as carriers for bioactive molecules such as exosomes [55]. Studies have shown that exosome-loaded hydrogels significantly enhance wound closure, collagen deposition, and angiogenesis compared to free exosomes, mainly due to improved stability and sustained release [56]. For example, alginate-based hydrogels and composite nanofiber–hydrogel systems have demonstrated the ability to prolong exosome activity and reduce their rapid diffusion from the wound site [57]. However, hydrogels alone may suffer from poor mechanical strength and rapid degradation, which can be improved through crosslinking or incorporation of reinforcing materials such as nanofibers [58].

Collagen, particularly type I and type III, represents a key structural component of the skin and is widely used in tissue engineering due to its excellent biocompatibility and ability to support cell adhesion and proliferation [26, 59, 60]. Collagen-based scaffolds have been shown to enhance extracellular matrix remodeling, promote fibroblast activity, and accelerate re-epithelialization [60]. Studies demonstrated that collagen sponges loaded with exosomes enabled sustained release, enhanced angiogenesis, and significantly accelerated full-thickness wound healing through modulation of inflammation and upregulation of healing-associated miRNAs such as miR-21-5p and miR-29a-5p [61, 62]. Additionally, advances in recombinant human collagen have reduced the risk of immunogenicity associated with animal-derived materials while maintaining structural integrity [63]. Composite systems, such as collagen combined with chitosan or incorporated into hydrogels, further improve mechanical properties, antimicrobial activity, and wound healing efficiency [64]. These findings highlight that integrating exosomes with collagen-based or hydrogel scaffolds represents a powerful strategy for enhancing full-thickness wound repair, which is consistent with the improved healing outcomes observed in scaffold-based approaches such as SIS in the present study.

Limitations

While providing encouraging histological data showing the effect of SIS–Exo on the development of granulation tissue, epidermal regrowth, angiogenesis, and collagen deposition, it is important to note some weaknesses in the design of the experiment. First, the experiment included an extremely low number of test subjects, which considerably reduced its statistical significance and increased the chances for Type II errors and overvaluation of results. Second, the limited time frame of the experiment

did not allow assessing such long-term effects as scar maturation, tissue remodeling, and recovery of functions of the regenerated skin, which was not assessed at all.

The experiment utilized one wound healing model (full-thickness dorsal excisional wound 1×1 cm in rats), and this is likely not to be representative of clinical wounds that could be chronic wounds, infected wounds, or large burn wounds. Lastly, using only one animal for the study reduces its generalizability, since murine wound healing is different from human wound healing (being mostly contraction-based) [65].

Furthermore, the absence of quantitative biomechanical testing (for example, tensile strength of healed tissue) reduces knowledge about functional tissue quality even with an improved histological structure. Besides, the study did not consider tracking of exosome residence time, release rate, and distribution in the SIS scaffold, which makes it difficult to evaluate how long exosomes retain their biological activity in vivo. It should be noted that there is no evaluation of possible immune or inflammatory reactions against SIS (porcine matrix) and exosomes in the combination. Overall, the low number of experimental groups and dose-response relationship in this study do not allow optimizing the SIS–Exo combination, and the lack of comparative studies with other advanced biomaterials and clinical dressings makes evaluation of the results more difficult. One more drawback of this study is that there was no assessment of wound closure kinetics performed. Although pictures of the wound at 14 days were provided to show clearly the state of wound healing (Figure S1-3), the area of the wound and the percent of wound closure could not be evaluated. It is recommended that future investigations be done using a standard method of digital planimetry and serial photography at different time points.

Future studies will be required to develop studies on increasing the number of experimental units and using more animal models with bigger wounds, such as full-thickness injuries and chronic wounds in pigs, which will increase translational value towards wound healing in humans. It is necessary to conduct long-term studies aimed not only at the assessment of wound closure but also at scar quality, tissue remodeling, and recovery of skin appendages, including hair follicles and sweat glands. Studies should be carried out to identify the underlying molecular mechanisms of SIS-exosome synergy, including analysis of signaling pathways for angiogenesis (VEGF), proliferation (Ki67), inflammation, and the ratio of type I and III collagen. Moreover, it is necessary to optimize dose, origin (MSC-derived exosomes and other cells), and loading efficiency of exosomes into SIS scaffolds, as well as to study exosome release kinetics and retention in vivo. Furthermore, focus should be on comparative studies of SIS-exosome systems with other clinically relevant biomaterials and dressings. Safety issues related to immunogenicity and inflammatory response to porcine-derived SIS and potential off-target effects of exosomes should be addressed.

Conclusions

SIS-Exo significantly enhances wound healing in a murine model. These results suggest that SIS-Exo is a promising therapeutic strategy for promoting skin wound repair and may have potential applications in

regenerative medicine. Further studies with larger sample sizes and longer observation periods are recommended to fully evaluate its efficacy and long-term outcomes.

Declarations

Ethical approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Research Ethics Committees of Laboratory Animals – Tehran University of Medical Sciences. (Date No. **IR.TUMS.AEC.1402.039**)

Clinical trial number

not applicable.

Consent for Publication:

Not applicable

Consent to Participate (Informed Consent): Human umbilical cord tissues were collected after obtaining written informed consent from the donors. The collection and use of these samples were approved by the Institutional Research Ethics Committee of Imam Khomeini Hospital (Approval No. IR.TUMS.IKHC.REC.1400.184). The animal experiments did not involve human participants; therefore, informed consent did not apply to the animal studies.

Competing Interests and Funding

The authors have no relevant financial or non-financial interests to disclose.

Author Contribution

MF, FTA, and RE contributed to the study conception and design. MF contributed to the study supervision, methodology development, investigation, data interpretation, manuscript preparation, and critical revision. FTA contributed to the experimental design, methodology, investigation, data analysis, interpretation of results, and manuscript writing, reviewing, and editing. RE contributed to the investigation, data analysis, interpretation of findings, literature review, and preparation, review, and revision of the manuscript. RA, AZ, and NS contributed to the experimental procedures, data collection, investigation, and manuscript review. MSR contributed to the study methodology, material design, validation of experimental procedures and results, supervision of research activities, analysis and interpretation of in vivo and in vitro data, manuscript preparation, critical revision, and final editing. All authors reviewed and approved the final manuscript and agreed to be accountable for all aspects of the work.

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Figures

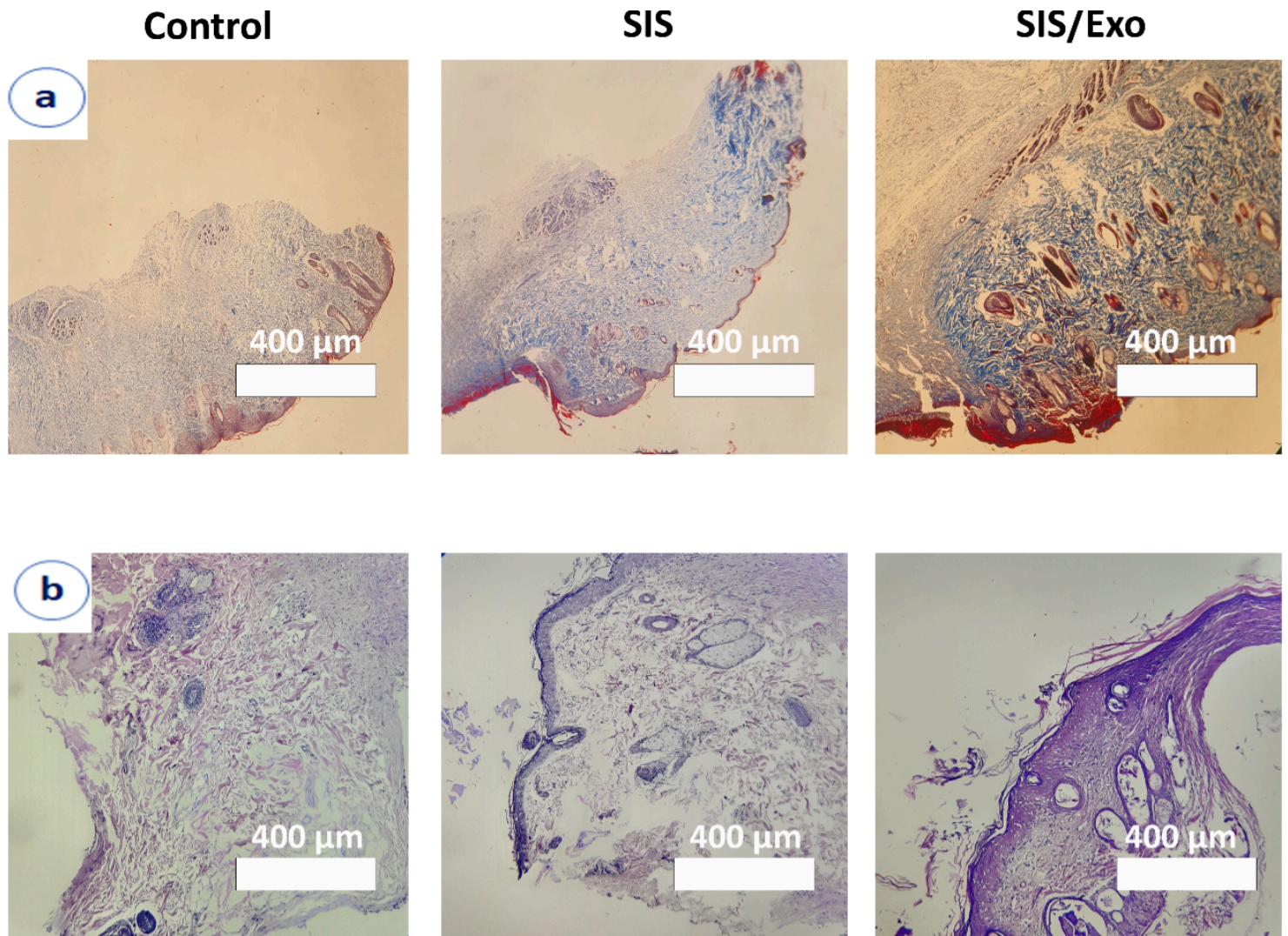


Figure 1

Histopathological analysis of control and interventional groups on day 14 post-operation. a) The trichrome staining of the Control, SIS, and SIS/Exo group on day 14 post-operation. b) The H&E staining of the control, SIS, and SIS/Exo groups reveals improved skin regeneration in the SIS/Exo group. SIS: Small intestine submucosa. SIS/Exo: Small intestine submucosa and exosome. Scale bar: 400 μm

Supplementary Files

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