Histopathological assessment of wound healing after using adipose-tissue derived mesenchymal stem cells with Tragacanth gum hydrogel and human amniotic membrane as dressing

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ABSTRACT

Wound healing and finding a solution for fast healing are among of the major issues of today's world. This study aimed to assess the effect of Tragacanth gum hydrogel as a three-dimensional scaffold of MSCs along with a wound dressing of human amniotic membrane in the healing of full-thickness skin wounds in rat. In this study, 54 Albino female rats (150 g) were divided into control, hydrogel, and hydrogel+stem cell groups. Under general anesthesia, two bilateral full-thickness wounds were created on the dorsal area by a 9.8-millimeter biopsy punch. Rats were euthanized on days 3, 10, and 21 for histopathology and cell tracking by PCR evaluation of tissue samples. The histopathological results showed that no significant difference was seen on days 3 and 21, and there were significant differences only on day 10. In terms of epithelialization, the treatment groups were significantly different from the control group hydrogel+MSCs had a statistically significant difference with the control group in terms of granulation tissue formation. Cell tracking results with PCR on days 3, 10, and 21 in the hydrogel+MSCs group showed that MSCs were found only on day 3. The results of the present study showed that the use of stem cells together with the Tragacanth gum hydrogel as a scaffold and the use of human amniotic membrane as a dressing can cause fast healing of full-thickness wounds.

Abbreviations

MSC: mesenchymal stem cell

PCR: Polymerase chain reaction

Introduction

*T*he skin acts as the first defense barrier of the body against external injuries, pathogens, and water loss, and it is also a thermoregulator [1,2]. In the management of wounds, the best method should be selected according to the size of the wound, the amount of tissue damage, the presence of infection, and the length of time since the wound was caused [3]. Naturally, the body's speed in responding to injury and wound healing should be a way that prevents the infection and severe inflammation or organ failure [4]. In addition, open wounds can be treated by two methods: primary (suturing) and secondary healing. Sometimes, due to the large size of the wound, the amount of tissue damage, infection, and economic conditions, the wound is treated with secondary healing methods (contraction and reepithelization), which would be a more appropriate and practical method [5]. Although a variety of local treatments with different substances are used for secondary wound healing, today, cell therapy is used as a new treatment with minimal side effects in the treatment of various diseases, including wound healing. Among the cells with different origins, MSCs have the potential to be used for wound cell therapy. MSCs are found in most tissues with different sources and have been used to manage the wound healing and regeneration process [6]. Studies showed that adipose-derived MSCs (AD-MSC) have effective properties such as easier separation, affordability, and lack of ethical issues, and they have high differentiation ability into different types of cells, including skin cells and myocytes [7].

The second solution that can be used in wound healing is the use of biological scaffolds as a carrier for the transfer of cells, drugs, medications and various compounds. One of these biological scaffolds is hydrogels, which can be made from various natural polysaccharides. Natural polysaccharides, as abundant polymers, are hydrophilic and environmentally friendly. They are more similar to the extracellular matrix and rarely cause inflammatory responses and cytotoxicity due to their structure and high hydrophilicity [8,9]. Tragacanth gum, as an anionic polysaccharide, is a natural polymer extracted from renewable sources of agrochemicals. Its advantages include wound healing and drug delivery to control and improve the microstructure, texture, stability, and viscosity of cosmetics, biological products, food, and pharmaceutical formulations. In addition, they are used as emulsifiers and thickeners [10–12]. Tragacanth gum hydrogels are hydrophilic polymers with three-dimensional structures that have a high capacity to absorb water and are used as a controlled drug release and wound dressing [13–15]. Moreover, they have biocompatibility features in the presence of different fluids and tissues of the body. They are similar to the native ECM in terms of the lack of toxicity, subtlety, non-allergenic power, and rubbery nature [16,17].

One of the protective dressings, that is widely used in wound healing, and its effectiveness has been confirmed in various studies, is HAM. The HAM triggers the migration and proliferation of the epithelial cells of the epidermis [18]. It has anti-inflammatory, antibacterial, and non-immunological properties and causes the wound to contract faster. It is impregnated with several essential growth factors [19].

Considering that all these valuable substances alone are effective in wound healing, in this study, the simultaneous effect of MSCs and Tragacanth gum hydrogel on wound healing and the use of HAM as dressing were investigated.

Results

MSCs Characterization and Differentiation

In passage 0, the cell colonies of MSCs were heterogeneously visible. As the passage numbers increased, the cells became more homogeneous and morphologically elongated (Figure 1A, B).

In this study, the cells were placed in the third passage in the differentiation media, inducing fat and bone cells. After 21 days of culture for differentiation into fat and 14 - 17 days for differentiation into bone, their successful differentiation towards fat and bone cells was proved by Oile Red O (bright red fat vacuoles) and Alizarin Red (Calcium ions deposited in the external matrix of cells) staining methods, respectively. Moreover, examining the activity of alkaline phosphatase was indicative of the differentiation of these cells to bone cells (Figure 1C-H).

DAPI staining of HAM

The results of this staining showed that cell removal was complete. The membrane was entirely uniformly black under the fluorescent microscope (Figure 2A-C).

Histological wound healing assessment

Epidermal cells regeneration

The process of wound healing 3 days after surgery in the rats of the control and hydrogel groups was observed only as the thickening of the epidermis tissue
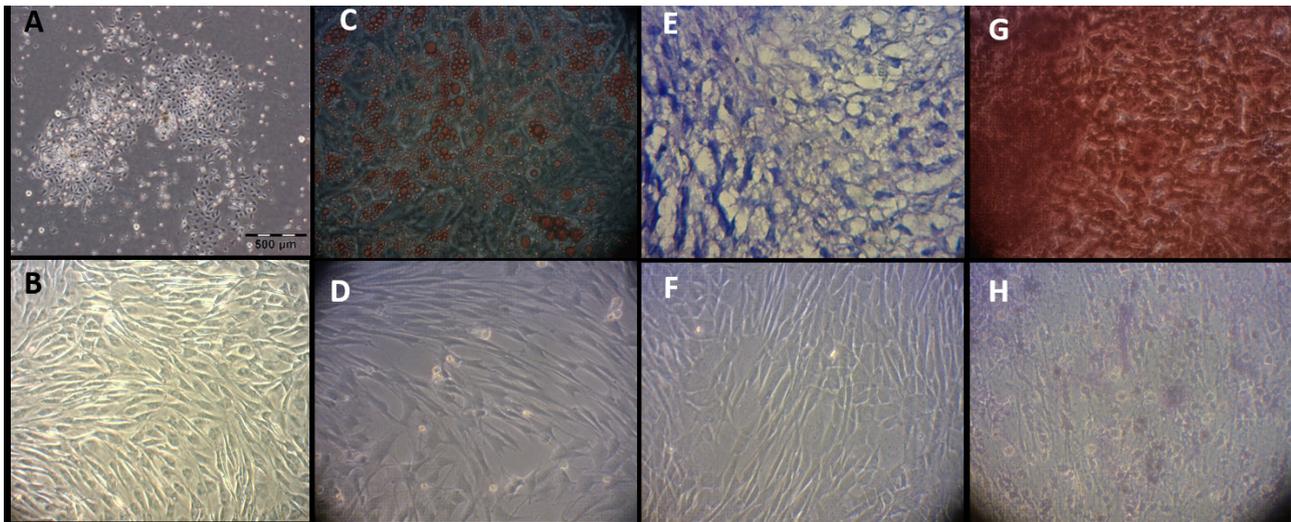
MSCs on scaffold in wound healing

Figure 1.
Characteristics of rat adipose tissue-derived mesenchymal stem cells (adMSCs). Phase contrast photomicrographs show the morphology of colonies of rat adMSCs at passage 0 (A) and expanded rat adMSCs at passage 1 (B). The cells represent spindle-like morphology. Rat adMSCs at passages 1-3 were characterized by adipogenic differentiation via Oil Red O staining that represents the lipid vesicles within differentiated cells (C) versus the control cell without adipogenic induction media (D). The osteogenic differentiation potential of rat adMSCs was also examined by Alkaline phosphatase activity assay (E) and Alizarin red staining (G) against their respective controls (F and H). All the images except A were taken by ×100 magnification.
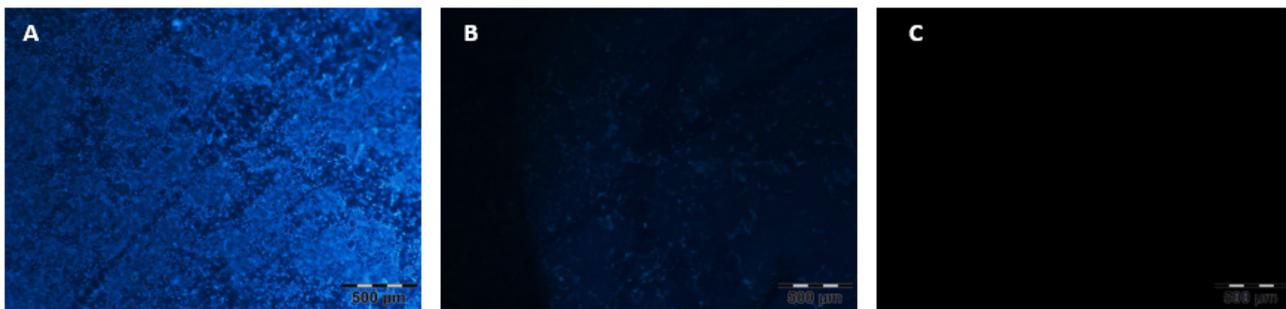

Figure 2.
Confirmation amniotic membrane decellularization by DAPI fluorescence dye. A: Amniotic membrane before decellularization, B: amniotic membrane during decellularization, C: amniotic membrane after decellularization. In this staining, the blue dots represent the nucleus of the amniotic membrane cells, and the black parts indicate the decellularized amniotic membrane.

at the cut edges. In some rats of the hydrogel+MSCs group, the epidermis was completely regenerated, and the entire wound was covered with keratinocyte cells. In addition, the amniotic membrane was observed as a serous layer covered with squamous cells on the wound scab in this group. In general, between the treatment groups and the control group were significant ($p < 0.05$) on the 10th day, and at this time, complete regeneration of the epidermis was observed only in the hydrogel+MSCs group (Figure 3A, 4A-D).

Inflammation response

According to our results, granulomatous inflammation was severe in all groups on days 3 and 10. On the 10th, in the hydrogel and control groups, the presence of more granulomatous inflammation and giant cells was confirmed. On the 21st day, the hydrogel+MSCs group had the lowest grade of granulomatous inflammation and inflammatory cells, but these differences were not significant ($p > 0.05$) (Figure 3B, 4E-G).

Granulation tissue formation

According to the total scores of granulation tissue formation on days 3 and 10, the samples of the hydrogel+MSCs group showed the highest rate of granulation tissue formation compared to the other groups, but the difference with the control group was significant only on day 10 ($p < 0.05$). On the 21st day, this criteria was higher in the hydrogel and control groups than hydrogel+MSCs but the difference was not significant (Figure 3C, 4H-J).

PCR analysis of male DNA in female tissue

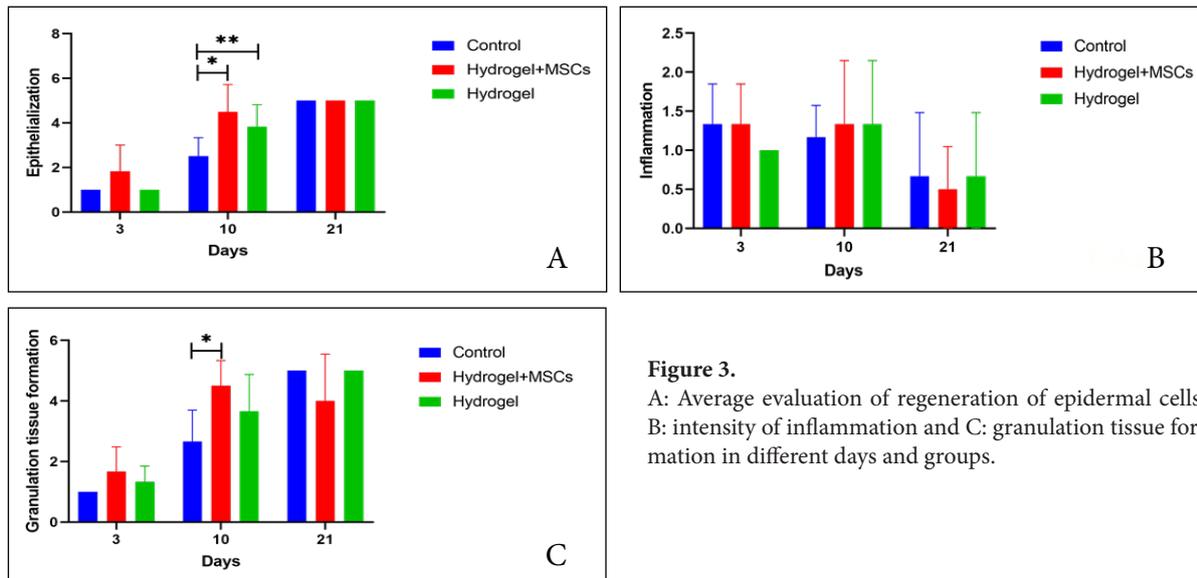Sex mismatch between donor and recipient an-

Figure 3.
A: Average evaluation of regeneration of epidermal cells, B: intensity of inflammation and C: granulation tissue formation in different days and groups.
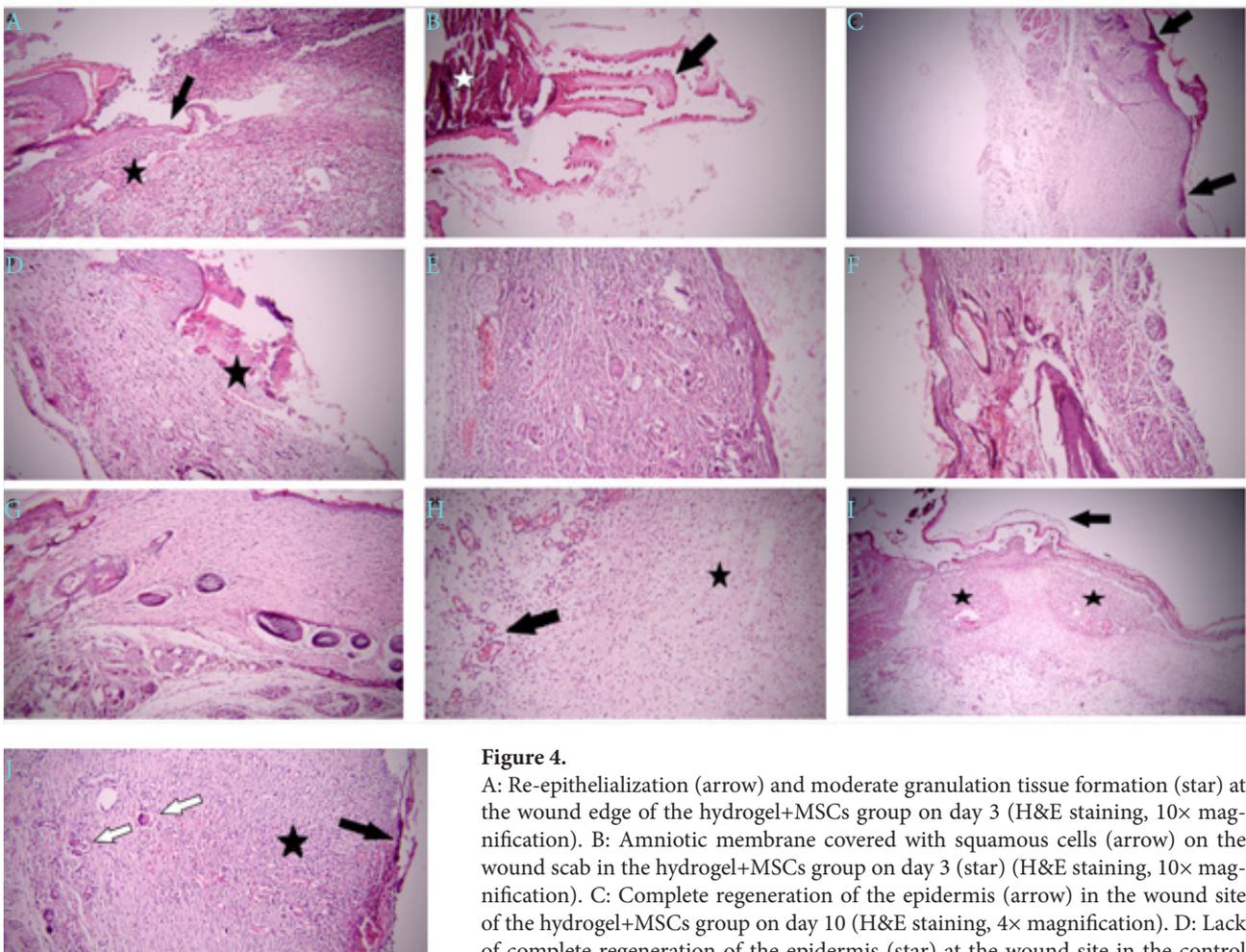

Figure 4.
A: Re-epithelialization (arrow) and moderate granulation tissue formation (star) at the wound edge of the hydrogel+MSCs group on day 3 (H&E staining, 10× magnification). B: Amniotic membrane covered with squamous cells (arrow) on the wound scab in the hydrogel+MSCs group on day 3 (star) (H&E staining, 10× magnification). C: Complete regeneration of the epidermis (arrow) in the wound site of the hydrogel+MSCs group on day 10 (H&E staining, 4× magnification). D: Lack of complete regeneration of the epidermis (star) at the wound site in the control group on day 10 (H&E staining, 10× magnification). E: Diffuse granulomatous inflammation in the dermis area at the wound repair site in the hydrogel+MSCs on day 10 (H&E staining, 10× magnification). F: Diffuse granulomatous reaction at the base of the wound in the hydrogel group on day 21. (H&E staining, 10× magnification). G: Granulomatous reaction around the unrecognizable structure in the control group on day 21. (H&E staining, 10× magnification). H: Hyperemia and the beginning of the angiogenesis process at the base of the wound (arrow) and the absence of granulation tissue formation (star) in the control group on day 3 (H&E staining, 10× magnification). I: Two foci of granulation tissue formation in the center of the wound (star) and the amnion membrane on the surface of the wound in the hydrogel+MSCs group on day 3 (arrow) (H&E staining, 4× magnification). J: Lack of complete regeneration of the epidermis and the presence of a scab on the surface of the wound (black arrow), the formation of abundant granulation tissue and angiogenesis (star), and the presence of granulomatous reaction in the base of the wound (white arrow) (H&E staining, magnification ×10).

imals was designed to determine the persistence of MSCs in the wound tissue. Female rats were injected with a hydrogel containing AD-MSCs of male rats at the wound site. The results obtained from PCR, revealed that MSCs were present until the 3rd day only in the wound of the group receiving hydrogel+MSCs. However, no bands were observed on the 10th and 21st days and in the two other groups (Figure 6).
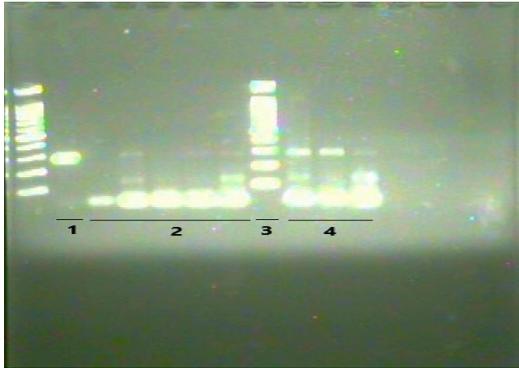

Figure 5.
Representation of the gel electrophoresis to trace the SRY gene in order to check the presence of MSCs in the wound healing site on day 3. From left to right: 1) MSCs isolated from the fat of male rats. 2) Samples were isolated from the hydrogel+MSCs group on day 3. 3) ladder. 4) Replication of three samples from the same group with higher DNA concentration.

Discussion

MSCs can differentiate into other cells and secrete or suppress the growth hormones or essential cytokines in the wound environment. AD-MSCs in large quantities are easily isolated and cultured and have great potential in therapeutic applications [20]. In the present study, the regeneration of the epithelium was completed on day 3 in the hydrogel+MSCs group, and in the other groups, it was completed on day 10. In different studies, the start of epithelialization was different, and most of them had significant differences with the control group. It has been shown in a research that AD-MSCs increase blood supply and the rate of granulation tissue formation in wounds, survive in the wound for up to 14 days, and have lasting effects on the wound [21].

Contrary to the above studies, one investigation showed that between the control and treatment groups, there was no significant difference in terms of the amount of collagen, epithelialization, angiogenesis, and number of fibroblasts and macrophages. They showed that AD-MSCs had a significant effect in reducing the size of the wound, but their effect on the severity of skin lesions and pathological factors was not confirmed. Compared to BMSCs (Bone MSCs),

they have a lower ability to differentiate into endothelial cells [22]. Furthermore, Karimi et al.(2014) reported that AD-MSCs had no significant improvement in acute burn wound healing [23].

The present study showed that the hydrogel+MSCs group had the highest amount of granulation tissue and angiogenesis on days 3 and 10, which decreased on day 21. Lotfi et al. (2019) stated that the granulation tissue thickness in the keratinocyte/MSCs/scaffold group rose in the first week, and declined significantly in the second week compared to other groups [24]. In the present study and the research by Lotfi et al., polymers made from natural materials such as hydrogels provided a suitable environment and direct cell contact. In the current investigation, the hydrogel group had the highest amount of granulation tissue on day 21 compared to the hydrogel+MSCs group, which could indicate the critical role of stem cells that have paracrine signaling properties, which reduce inflammation, and promote angiogenesis and cell proliferation [24].

In this study, we used allogenic AD-MSCs. According to the literature, autologous MSCs have more accelerated cicatrization than allogeneic MSCs. However, in burn injuries, allogeneic MSCs can be the only available option [25]. Research showed that the intradermal injection of allogenic AD-MSCs in burn wounds caused a significant difference on the 14th days with the control group [16].

In the detection of the SYR gene by PCR, the band of this gene was observed only on day 3 in the hydrogel+MSCs group. Hanson et al. (2016) injected allogenic male AD-MSCs intradermally in the partial-thickness of female minipigs. In female tissues, male DNA content was evaluated by the PCR amplification of a 377 bp segment from chromosome Y. They observed Y chromosome bands with a decreasing trend on days 0, 7, and 10 [26]. Based on this study, it would have been better to include the cell tracking investigations of day 7 in the present study to better understand this decreasing process . The difference between the present study and the above study may be attributed to the type of animal modeling, method, and amount of stem cell injection. The reason for the decrease in the presence of MSCs in the wound site is unknown. However, it can be because of MSCs migration from the wound site, MSCs phagocytosis by macrophages, or mechanisms involved in cell processing and tissue regeneration [26].

Tragacanth gum hydrogel can be a suitable scaffold for AD-MSCs. It accelerates the proliferation and differentiation of cells and provides a suitable space for the support and adhesion of cells. It is also capable of expressing genes for up to 21 days and maintains the original morphology of cells. In the present study,

the hydrogel and control groups had significantly different epithelialization on day 10. Although no significant difference was observed in inflammation and granulation tissue formation between the hydrogel and other groups, on the 10th and 21st days, the rate of granulation tissue formation in the hydrogel group was higher than in other groups. One of the reasons is the high concentration of hydrogel, and the reduction of inflammation in the stem cell group is may result from the presence of MSCs. A study similar to the current research showed that wound closure occurs faster in the PCL-GT-stem cells group than in the PCL-GT scaffolds group. Granulation tissue, collagen synthesis, and angiogenesis were improved in the PCL-GT-stem cells group. They stated that GT accelerates the transition from the inflammatory and germinal phases as well as the maturation of scar tissue [27–29]. Researchers demonstrated that creams made from Tragacanth gum at a concentration of 6% had the highest effect on rabbit wound healing compared to the control group [27]. In another study, with the daily application of Tragacanth gel, a significant difference was observed in terms of epithelialization, inflammation, and granulation tissue on the 10th day compared to the control group. Similar to the present study, they showed that Tragacanth gum caused most of the wounds to close on the 10th day by accelerating wound contraction [28].

A proper dressing should enhance epithelial regeneration, control the amount of exudate, prevent material leakage, reduce inflammation and infection, and be comfortable for the patient. In this study, amniotic membranes, as an economically reasonable alternative biomaterial were used to benefit from the above characteristics and also prevent hydrogel leakage. In some studies, the use of amniotic membranes alone in wound healing was ineffective [30], but in others, it had no significant difference with the control group or other treatment groups [31]. Studies have shown that using the amniotic membrane alone is effective for shallow wounds while a more effective solution is required in wide and deep wounds, such as full-thickness wounds and third-degree burns [32].

Studies showed that the application of MSCs with cellular/acellular amniotic membrane multiplies the rate of wound healing compared to utilizing amniotic membrane alone [33]. In this study, on the 3rd days in the stem cell group, acellular human amniotic membrane was observed as a serous layer covered with squamous cells on the wound scab, which is a sign of the effective role of MSCs.

In summary, in this study, the synergism effect of MSCs, Tragacanth gum hydrogel, and human amniotic membrane as a dressing was investigated. Histopathology results showed that the combination of SCs

and Tragacanth gum hydrogel was influential in the immediate wound closure, and the human amniotic membrane played a supporting role.

Materials and Methods

Ethical statement

All procedures and experiments were performed on animals according to the guidelines of the Animal Care Committee and were approved by the Research Ethics Committees of Ferdowsi University of Mashhad (IR.UM.REC.1400.333).

Isolation and Cell Expansion of ADSCs

Adipose tissue source was prepared from a male Wistar rat (8 weeks old). After general anesthesia with ketamine 10% (50 mg kg-1, Bremer Pharma GmbH, Warburg, Germany) and xylazine 2% (5 mg/kg, Alfasan, Woerden, Netherlands), laparotomy was performed under aseptic conditions. A volume of. 2-3 ml of accumulated fat from the retroperitoneal, perirenal, and inguinal regions was dissected bilaterally and collected with at least manipulation. These samples were transferred to the laboratory in sterile conditions and in the shortest time for the subsequent steps. Adipose tissue was washed with PBS (Gibco, Eggenstein, Germany) containing 1% of the penicillin–streptomycin (Gibco, Eggenstein, Germany) to separate the vessels and connective tissues attached to the fat pieces as much as possible and the process was repeated if necessary. For enzymatic digestion, 1 mg/ml of collagenase type IA [Sigma-Aldrich (St. Louis, MO)] and 10 mg of bovine serum albumin (Invitrogen (Carlsbad, CA)) were dissolved in PBS. After adding 2 mM CaCl2 to this solution, it was sterilized using 0.2 micron filters (Jet Biofil/ Orange, Canada). The collagenase solution was added to 3 ml of adipose tissue fragments obtained in the previous step. The mixture of fat and collagenase was incubated at 37°C for 1 h in a shaking water bath (Memmert. Germany). After the enzyme digestion step, the adipose tissue was diluted at 1:3 with PBS and was centrifuged (800g, 10 min). After centrifuging, four phases were visible. Next, the suspended fats fragments, adipose tissue, and PBS in the three upper phases were removed from the test tube with a pipette without disturbing the cells. In the DMEM / low glucose medium (DENAzist Asia's DMEM-LG (Low Glucose)), the cell pellet was resuspended, and centrifugation (at 400 g for 6 min) of the solution was done. The Sediment (the pellet, which contained MSCs) was resuspended with DMEM/LG and cultured in DMEM-LG with 10% fetal bovine serum and 1% penicillin/streptomycin. Then, it was transferred to a 75T flask and maintained at a temperature of 37°C with 95% humidity and 5% CO_2. After 48 hrs, the culture medium was replaced and changed each day until the confluence of cells in the flask reached 80-90%. Then, the previous culture medium was removed, and cells were washed with PBS. Cells were then detached by the application of trypsin–EDTA for subculture [24,34–37]. Briefly, after adding trypsin to the flask, it was incubated for 5 minutes, a new culture medium containing 10% FBS was added to the flask to neutralize the effect of trypsin, and the contents of the flask centrifuged at 400 g for 6 minutes. The supernatant medium was removed, a new medium was added to it, and several times pipetting with a sampler was done until the cells were suspended. After cell counting using trypan blue, 75×10^4 cells were transferred into a new 75T flask, and after adding culture medium, the flask was transferred to the incubator.

Characterization of MSCs

The capability of the cells to differentiate into osteoblasts and

adipocytes was analyzed. Flow cytometry was performed in the previous study to assay the immunophenotype [24].

Adipogenic and Osteogenic Differentiation of MSCs

AD-MSCs 80-90% confluency, after 3 passages, were cultured in the adipogenic differentiation medium (DMEM supplemented with 10% FBS, 100 μmol/L indomethacin, 10 mM β-Glycerol phosphate, and 1 μM dexamethasone) (Sigma, Germany)) for 21 days. After changing this medium, at an interval of 2-3 days, the cells were stained with Oil red O at the end of this differentiation period. Also, for osteogenic differentiation, the cells were cultured in the osteogenic differentiation medium (DMEM supplemented with 10% FBS, 10 mM ascorbic acid, 10 mM β-Glycerol phosphate, and 0.1 μM dexamethasone) (Sigma, Germany)) for 14-17 days and then this induction medium was refreshed at an interval of every 2-3 days and cells were stained with Alizarin red solution. All these steps were performed on MSCs without induction by differentiation medium as a control. After staining, the cells were observed under the iX70 inverted microscope (Olympus, Japan)[37].

Alkaline phosphatase assay

The level of alkaline phosphatase activity increases with the differentiation of MSCs into osteoblast cells. Therefore, alkaline phosphatase activity can be used as an indicator to investigate the differentiation of these cells that can be easily detected by the substrate of this enzyme, i.e. BCIP/NBT. Briefly, after differentiation, the cells were washed with PBS buffer and fixed in 4% paraformaldehyde. The paraformaldehyde solution was removed and washed, then a BCIP/NBT tablet ((5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) (Roche, USA) was dissolved in 10 ml of deionized water and 1 ml was poured on the differentiated cells and the control group and kept at room temperature for 5 to 10 minutes. For better penetration of the substrate into the cells, 0.05% Tween-20 was added to the PBS buffer. Then,

they were washed with PBS buffer and examined with an inverted light microscope [37].

Preparation of Tragacanth gum hydrogel

To prepare Tragacanth gum hydrogel 6 % (6g/100 mL), after passing sterilization steps under the hood and UV lamp, 1/5 g of Tragacanth powder (Missouri, United States) is mixed with 25 ml of culture medium of DMEM-LG containing FBS and then it was shaken for at least 2hrs.

Preparation of Human Amniotic Membrane (HAM)

The Ethics Committee of the Ferdowsi University of Mashhad approved the use of HAM for this research. After obtaining written informed consent, placentas were obtained during elective cesarean section. Viral diseases were negative for all donors in the serological results. DMEM; Gibco, Scotland contained 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B solution (Biosera UK) was used to place the placental tissue in it. The placenta was washed several times in PBS with an antibiotic/antimycotic solution. Blunt dissection was performed to detach the HAM from the chorion. HAM was divided into approximately 2.5 × 2.5 cm pieces. To perform de-epithelialization, 0.5 M NaOH (Sigma-Aldrich) was used with a cotton-tipped applicator to rub the cells, followed by washing with sterile PBS in a shaker for 10–15 min. To confirm the cell removal of the amniotic membrane, DAPA staining (2μg/ml PBS) was done [38,39].

In vivo wound healing experiments in an animal model

In total, 54 female rats aged 1.5 months and weighting ~150 g were randomly divided into control, Tragacanth gum hydrogel (hydrogel) and Tragacanth gum hydrogel+MSCs (hydrogel+MSCs) groups. The rats were anesthetized by an intraperitoneal injection of 50 mg/kg ketamine and 5 mg/kg xylazine. To evaluate
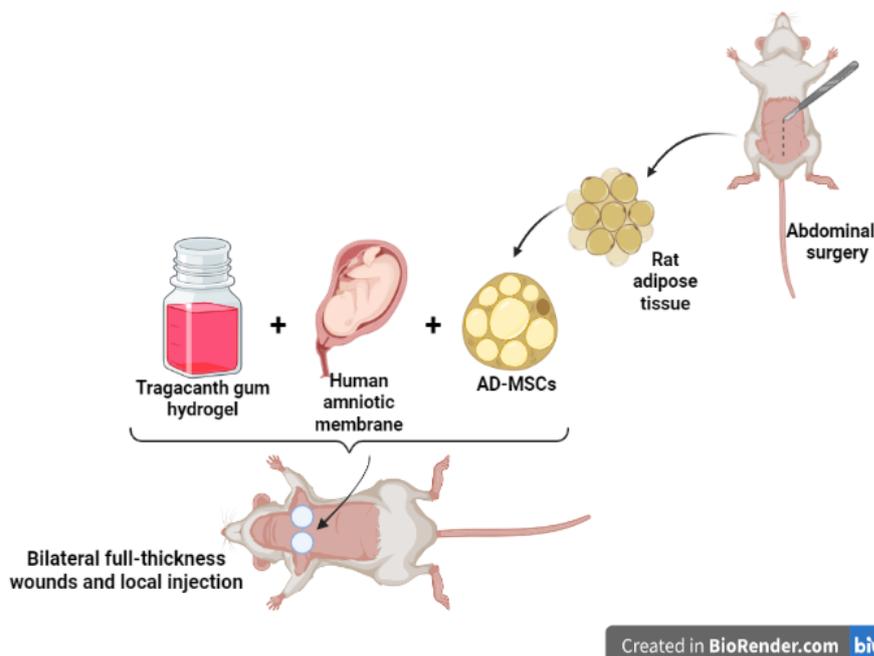

Figure 6.
Schematic representation of the study. Created with BioRender.com.

the effects of these compounds on wound healing, two full-thickness circular wounds of equal sizes (8.7×8.7 mm2) were aseptically generated using biopsy punches on either side of the dorsal area of each rat. The rats in the control group were treated with a hydrofilm dressing; in the hydrogel group, the amniotic membrane was sutured on the wound, 0.1 ml hydrogel was injected, and hydrofilm dressing was placed on them. In the hydrogel+MSCs group, following the suturing of the amniotic membrane, 0.1 ml gel-containing cells (3×10^5) were placed on the wound, and finally, the hydrofilm dressing was glued on the set (Figure 6).

During this research, the rats were kept in the animal house. They were placed individually in cages a temperature of 24˚C and a cycle of 12 hrs of light and 12 hrs of darkness with free access to water and food.

Histopathology

On days 3, 10, and 21 after surgery, six rats were selected, and after euthanasia with chloroform, sampling was conducted under sterile conditions. One of the two wounds was selected randomly, and the skin wound and surrounding skin were fixed in 10% formaldehyde, embedded in paraffin, and cut into 5 μm sections. Standard H&E staining was performed, and the samples were evaluated quantitatively and qualitatively in terms of epithelium of regeneration of the epithelium, the number of inflammatory cells in the area, and the amount of granulation tissue formation. The evaluated factors were scored as follows: regeneration of the epithelial tissue (beginning of the regeneration of the epithelial tissue or thickening of the cut edges = 1, coverage of less than half of the wound = 2, coverage of more than half of the wound = 3, coverage of the entire wound with low thickness = 4 , coverage of the entire wound with keratinization and normal thickness = 5); degree of inflammation (absence of inflammation = 0, mild inflammation = 1, moderate inflammation = 2, severe inflammation = 3); degree of formation and maturation of granulation tissue (absence of granulation tissue = 0, low granulation tissue formation = 1, moderate granulation tissue formation = 2, abundant granulation tissue formation = 3, abundant granulation tissue formation and with tissue maturity = 4)[40].

PCR

To evaluate the persistence of MSCs injected in the local tissue wound bed, tissue samples were taken in sterile conditions and were frozen (-80˚C). As a pre-extraction step, these sections were crushed in liquid nitrogen using sterile pestles and mortars, and the obtained powder was used for DNA extraction. Genomic DNA was isolated using the Animal DNA Isolation Kit (DENAzist Asia Co., Mashhad, Iran) according to the manufacturer's instructions. To trace the MSCs of male rat adipose used in the wound site, the Y chromosome was selected by the SYR gene [41]. Because living rats are female, the mentioned gene could be traced. Primers that were used to amplify the SRY gene (PCR product size: 273 bp; Gene ID: 103694554) were 5'- GTAGGTTGTTGTC-CCATTGC-3' and 5'-GAGAGAGGCACAAGTTGGC-3'. The PCR protocol consisted of an initial denaturation step at 94°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 20 sec. A final extension of 5m at 72°C was applied at the end of the PCR reaction. The extracted DNA was quantified by a spectrophotometer at wavelengths of 260 nm and 280 nm. After DNA extraction, a 1.5% agarose gel was used, which consisted of 0.75 g of agarose powder, a solution containing 1 ml of TAE buffer, 49 ml of distilled water, and 1μL of ethidium bromide. For preparing of the Master mix solution, 20 ng per PCR sample was taken from the extracted DNA, and the final volume was adjusted to 10.5 μL with distilled water. The samples were then spun, and 14.5 μL of the Master mix and the primer solution were added to each sample. After another round of spinning, the samples were ready for use.

Statistical analysis

Statistical analyses were performed using SPSS (Ver. 26). Appropriate tests (Kruskal-Wallis Test and Mann–Whitney U-test) were used to analyze the data. $p < 0.05$ was considered significant.

Authors' Contributions

Conceptualization and Methodology: Hossein Kazemi Mehrjerdi, Hojjat Naderi-Meshkin, Hossein Nourani, Jeiran Rahvarian; Formal analysis and investigation: Hossein Kazemi Mehrjerdi, Shiva Amanollahi; Writing - original draft preparation: Shiva Amanollahi, Jeiran Rahvarian; Writing - review and editing: Shiva Amanollahi and Hossein Kazemi Mehrjerdi; Funding acquisition and Supervision: Hossein Kazemi Mehrjerdi, Hojjat Naderi-Meshkin.

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Competing Interests

The authors have no financial conflicts of interest.

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Online supplemental material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.