



# Effective Combination Therapy with Human Amniotic Membrane Mesenchymal Stem Cells and Low-Level Laser Therapy in Wound Healing in Animal Models

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## Abstract

**Introduction:** Many studies have recognized the importance of new methods in wound healing. This study aims to investigate the healing effects of allograft extra embryonic mesenchymal stem cells (MSCs) with and without low-level diode laser irradiation when grafted into full-thickness skin defects in diabetic animal models.

**Methods:** In this experimental study, in order to make the rats diabetic, we used an intra-peritoneal injection of streptozotocin. Human amniotic membrane derived mesenchymal stem cells (hAMSCs) were irradiated with a low-level diode laser. Two full-thickness excisions were made on the backs of the rats. Next, the rats were divided into the following groups: group 1: low-level laser (LLL) irradiated hAMSCs and group 2: hAMSCs alone transplanted into skin wound. Histopathologic, ultrasound and elasticity evaluations were performed 7, 14 and 21 days after grafting.

**Results:** In the evaluated rats, epithelial formation was on day 7 and increased until day 14. On days 7, 14 and 21, the percentage of epithelial formation in the irradiated cell group was significantly higher than that in the cell group, so that, on day 21, the epithelium in this group completely covered the wound surface while in the control group the wound surface was still not completely covered. In terms of angiogenesis, on day 7, the irradiated cells were significantly lower than the cells. Also, the formation of collagen in the cellular hydrogel group could confirm the effectiveness of amniotic MSCs in collagen production and thus accelerate the wound healing process. In comparison with hAMSCs alone, irradiated hAMSCs increased the thickness and elasticity of the skin.

**Conclusion:** Low-power laser along with MSCs can be effective in improving chronic wound condition in the animal model.

**Keywords:** Mesenchymal stem cells, Allograft, Diode lasers, Chronic wounds, Low-level laser.

## Introduction

Chronic wounds have a gradual onset and their healing process has stopped due to some factors such as diabetes, lack of proper blood supply, and local pressure, and the inflammatory stage of wound healing has been prolonged (more than four weeks) despite major improvements. A common but serious consequence

of prolonged uncontrolled diabetes mellitus (DM) is diabetic foot ulcers (DFUs), while the major cause of non-traumatic lower extremity amputations is diabetic foot infections (DFIs) of DFUs that led to about 73 000 amputations in the US in 2010. Moreover, the associated cost of DFIs is approximately \$176 billion each year. In wound healing, some efforts were made to find effective

methods for better wound healing in the shortest possible time and with minimal complications.<sup>1</sup> Protective and therapeutic approaches to stimulating healing include the application of wet absorbent dressings, bioengineered gauzes, vacuum-assisted closure, Regranex PDGF gel, and unloading. Nevertheless, none of these approaches has consistently demonstrated an advantage, and the cure of chronic wounds is a prolonged and difficult process.<sup>2</sup> Stem cells promise an emerging opportunity for advancing tissue repair and regeneration. Mesenchymal stem cells (MSCs) regulate the main phases of normal wound healing. Although MSCs may differentiate in the wound, it has been shown that they enhance wound healing through multiple effects, including modulation of inflammation, promotion of angiogenesis, and stimulation of cell movement during epithelial remodeling. The immunosuppressive properties of MSCs allow their potential use in allogeneic therapy.<sup>3</sup>

Low-level laser therapy (LLLT) as a therapeutic modality was introduced by Mester et al, who noted an improvement in wound healing with the application of a low-energy (1 J/cm<sup>2</sup>) ruby laser.<sup>4</sup> The application of LLLT was shown to enhance wound healing in animal models and in clinical trials.<sup>5</sup> In wound irradiation, several studies used visible or IR laser light due to their effects on the wound healing process and pain relief.<sup>6</sup> At the cellular level, because the fibroblast plays a crucial role in wound healing, most of the studies published in the LLLT literature have examined the effect of LLLT on fibroblast cell growth, locomotion, and production of collagen.<sup>7,8</sup> Although the studies appear to show an increase in collagen deposition and proliferation of fibroblasts, endothelial cells, and keratinocytes after exposure to LLLT, most of the published results are conflicting. These discrepancies may be attributed to disparities in the study design, including the use of different lasers, variations in parameters, and differences in cell culture techniques.<sup>9</sup> The use of a combination of therapeutic agents and biological stimulators could assist with the treatment of non-healing wounds and show a synergistic effect to improve the success of healing in different wound models.<sup>9,10</sup> Some studies investigating the laser as an alternative or adjunctive method for surgical wounds have shown its efficacy in enhancing surgical closure, reducing pain and infection, and shortening the period of wound healing.<sup>11-13</sup> Different parameters and irradiation conditions like exposure time, frequency, and duration of treatment influence the treatment outcome.<sup>14</sup> Therefore, it is necessary that researchers recognize the appropriate selection of these parameters in different studies to develop a successful treatment. The new concept of this paper is to investigate the effects of low diode laser irradiation with allograft extra embryonic MSCs on the healing of chronic wounds in animal models.

## Materials and Methods

### Placenta Tissue Collection

Placenta tissue was obtained from Mahdijeh hospital in Tehran, Iran, with the consent of mothers aged between 20 and 40 years after elective cesarean section.

### Animals

All the techniques were accepted by the Medical Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran. Twenty 3-month-old Sprague-Dawley male rats that weighed approximately 220 g were used. The rats were kept in standard plastic cages cleaned and ventilated daily. Type 1 DM was induced in all rats. For all animals, two full-thickness excisions using a sterile punch were made on the skin on their backs.

### Diabetes Induction

The diabetes model was induced in rats by injecting a dose of 50 mg/kg streptozotocin intraperitoneally. Three days after injection, the symptoms of diabetes such as binge drinking and urinary incontinence appeared in the rats. Glucose levels in the blood sample taken from the tail were measured using a glucometer 4 days after injection. Rats with blood glucose higher than 246 mg/dL were considered diabetic rats.

### Isolation and Culture of hAMSCs

Human amniotic membrane tissue was randomly received from 30 donors. Complete placental samples were received in a sterile receiver in the operating room after cesarean section from women aged between 20 and 40 years. The mean gestational age was 39 weeks. In the tissue laboratory, it was placed on a sterile tray and its various components were isolated for cell culture in those areas and placed in a Falcon tube containing 30 cc of phosphate-buffered saline (PBS) containing antibiotics (2X or 3X) (100 mg/mL). All separation and culture steps were performed under a sterile air hood cover in accordance with good cell culture practice (GCP) instructions in the culture room.

In the amniotic membrane tissue plate, detached chorionic tissue was removed. The tissue was completely cut individually with scissors and transferred to a falcon tube containing PBS. Centrifugation (1750 RPM and 7 minutes) was performed. The supernatant was drained, and about 25 cc of collagenase (1 mg/mL) (2 to 3 times the tissue volume) was added to 10 cc of the tissue inside each tube and incubated for 2 hours. After this period, the enzyme activity was neutralized by adding an equal volume of 10% culture medium. The contents of the Falcon were passed through a funnel. Some PBS was poured onto the strainer to collect all cell contents. Centrifugation (1750 RPM and 7 minutes) was performed. Cell pellets were taken in the DMEM low glucose medium containing 20% fetal bovine serum (FBS) serum for culture in 25 flasks. The cells were evaluated daily by an inverted microscope

(Olympus IX70) from the day after isolation.

To propagate the culture medium, we drained it into a flask or plate and poured some PBS on the cells. PBS was also drained, and about 1-2 cc of trypsin 0.25% was added and incubated for 5 minutes. Cells were counted by a hemocytometer and trypan blue solution.

#### ***Evaluation of MSCs Specific Markers in hAMSCs***

To confirm the origin of MSCs, we used flow cytometry analysis to assess surface markers and identify the phenotype of the stem cells isolated from the amniotic membrane. In the first step, after separating the cells from the flask, the cells were centrifuged (RPM 1500) and washed for 5 minutes by adding 2 mL of PBS solution to the cells. After washing, the final volume of cell precipitate was increased to 1 mL by PBS. The flow cytometry tube was prepared and 500  $\mu$ L of cell suspension was added to each. Monoclonal antibodies were then added to the tubes separately (CD34-PE, CD45-FITC as negative markers and CD44-FITC, CD73-PE, CD29-PE and CD166-PE as positive markers). After shaking the tubes, they were incubated for 30 minutes at 4°C. Then 500  $\mu$ L of PBS solution was added to the tubes for rinsing and centrifuged at 1500 RPM for 5 minutes. 250  $\mu$ L of PBS solution was added to the cell precipitate, and the sample was read in a flow cytometer. Finally, the data obtained were analyzed by software.

#### ***Evaluation of Differentiation Potential in hAMSCs***

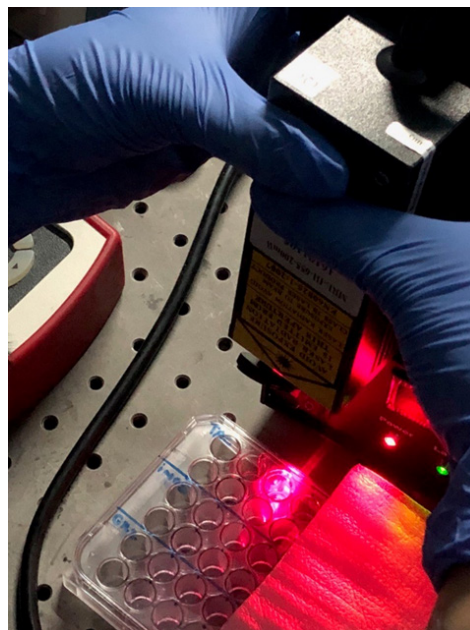
The differentiation potential of cells as mesenchymal cells was investigated.  $5 \times 10^4$  cells were cultured for 21 days in a 24-well plate containing a differential culture medium. Adipocyte differentiation media (Invitrogen) and Osteoblast differentiation media (Invitrogen) were used to differentiate into adipocytes and osteocytes respectively. Then on day 22, Oil-Red dye was used to evaluate fat differentiation, and Alizarin-Red dye was used to evaluate bone differentiation.

#### ***Evaluation of Viability in hAMSCs***

The Annexin V kit (BD Pharmingen, USA) was used to evaluate the viability and apoptosis rate in human amniotic membrane derived mesenchymal stem cells (hAMSCs) before transplantation. After washing with PBS and centrifugation (5 minutes, 300 RPM), 100  $\mu$ L of the annexin binding buffer was added to  $10^6$  cells. Then, annexin V and propidium iodide (PI) were added and placed at room temperature and in the dark for 15 minutes. Then 400  $\mu$ L of the connection buffer was added to the tubes, and the reading was conducted by flow cytometry.

#### ***Laser Radiation of hAMSCs***

The hAMSCc were treated with a low-power diode laser (660 nm) and a power of 1-5 MW, 3 times at two-day intervals (Figure 1).



**Figure 1.** Low-Level Laser Radiation on Human Amniotic Membrane Derived Mesenchymal Stem Cells.

#### ***Development of a Diabetic Wound Model in Animals***

After developing diabetes and measuring blood glucose, we anaesthetize the mice and washed and shaved their backs. The area was then disinfected with alcohol and betadine, and then a sterile puncture wound was made by removing the entire thickness of the skin, including the epidermis and dermis, with a sterile punch. The wound site was then cleansed with sterile gauze and normal saline. The day of surgery was considered as day 0.

#### ***Transplantation of hAMSCs***

Rats were randomly divided into 2 groups. The first group received only MSCs and the second group received diode laser-irradiated stem cells in their wounds. 21 animals were placed in each group. Animals in each group were divided into three groups on days 7, 14 and 21. 200 000 cells were injected into each wound, and then Mepitel dressing (Mölnlycke) was placed on the wound and covered with clear adhesive.

#### ***Photography and Evaluation of Wound Closure***

The appearance of the wound and its closure on different days (0, 7, 14 and 21) were done by photography in order to evaluate the healing and closure rate. Dressings were removed after animal anesthesia in order to capture images of the wound area on different days, and imaging was conducted under the same and standard conditions.

#### ***Histopathologic Examination***

The skin tissue including the wound and the surrounding area was removed by 2 cm and placed in a 10% formalin solution. Formalin-fixed samples were placed in baskets with the number of each rat. H&E staining, Masson's

trichrome staining, and elastic staining were done in a standard manner. Prepared tissue samples were evaluated for epithelial regeneration, angiogenesis, number of fibroblasts, number of neutrophils and inflammation, macrophages and keratin layer (in H&E staining), collagen fibers (in mason trichrome staining), and elastic filaments (in elastin staining) with a  $\times 40$  magnification.

### Wound Ultrasound Evaluation

The animals were transferred to a skin biometrics laboratory on days 7, 14 and 21 to evaluate changes in the thickness (thickness or depth) and density of the dermis and epidermis of the wound area. Animals were anesthetized, and the wound area was examined with a 75 MHz ultrasound probe (MHZ) (type DUB-USB75 model from TPM, Germany).

### Wound Elasticity

To evaluate the elastic changes of the wound skin, we anesthetized the animals on days 7, 14 and 21 and examined their wound area with a Cutometer (Dual MPA 580, Courage + Khazaka Company, Cologne Germany).

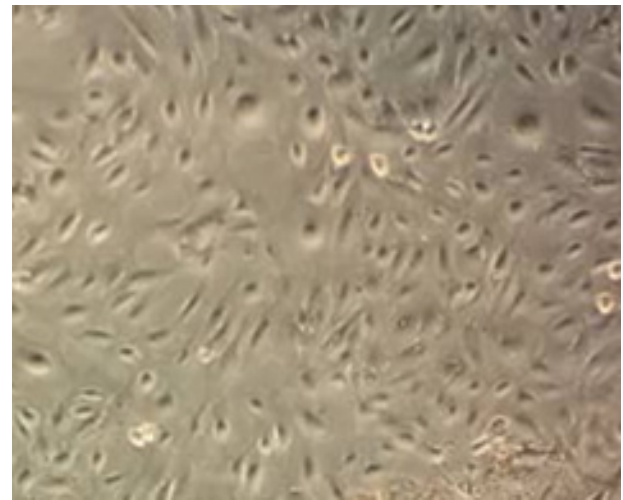
### Statistical Analysis

An independent-samples *t* test was used to compare the parameters of wound healing between the two groups on different days separately, and a one-way analysis of variance was used to compare the changes in the tested groups on days 0, 7, 14 and 21. The significance of differences between the groups was considered at the level of  $\alpha < 0.05$ .

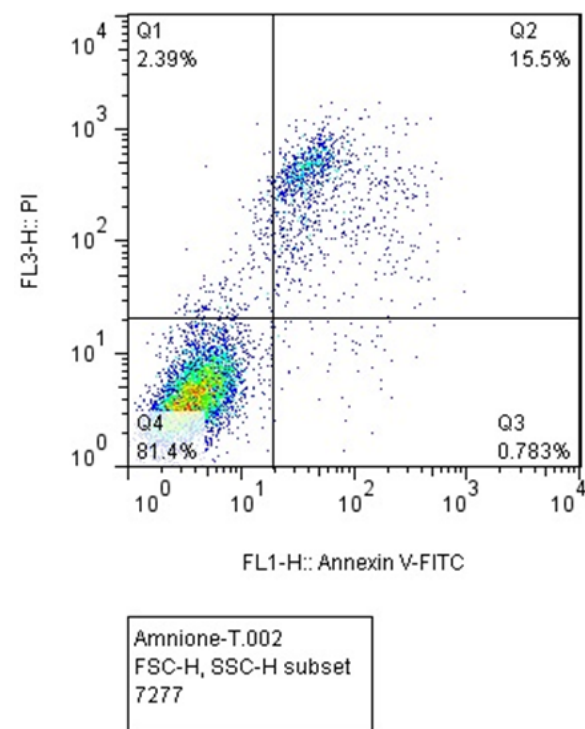
## Results

### Morphology and Proliferation of hAMSCs

The results showed that the isolation process should be performed no later than a few hours after cesarean section. Storing the sample for a day or more caused contamination and the desired result of separation was not obtained. Chopping the tissue was suitable in the enzymatic method, in which the enzyme worked more easily. Incubation at 37 degrees for at least 2 hours was appropriate, and in the meantime, a mechanical pipetting was helpful. The enzymatic method required less time to reach the cells. The morphology of the cells was spindle-shaped in the confluent state and elongated in the sub-confluent state. In reverse microscopy, 7 days after the initial culture of the skin, sticky, spindle-shaped fibroblasts were observed radially around the tissue (Figure 2). Cells in passage 3 were used for tissue grafts. Cell counts showed that each T75 flask contained about 2 million cells. The evaluation of apoptosis in cells showed that a high percentage of cells in passage 3 were alive and a small number of them had apoptosis and were therefore suitable for transplantation for wound healing (Figure 3).



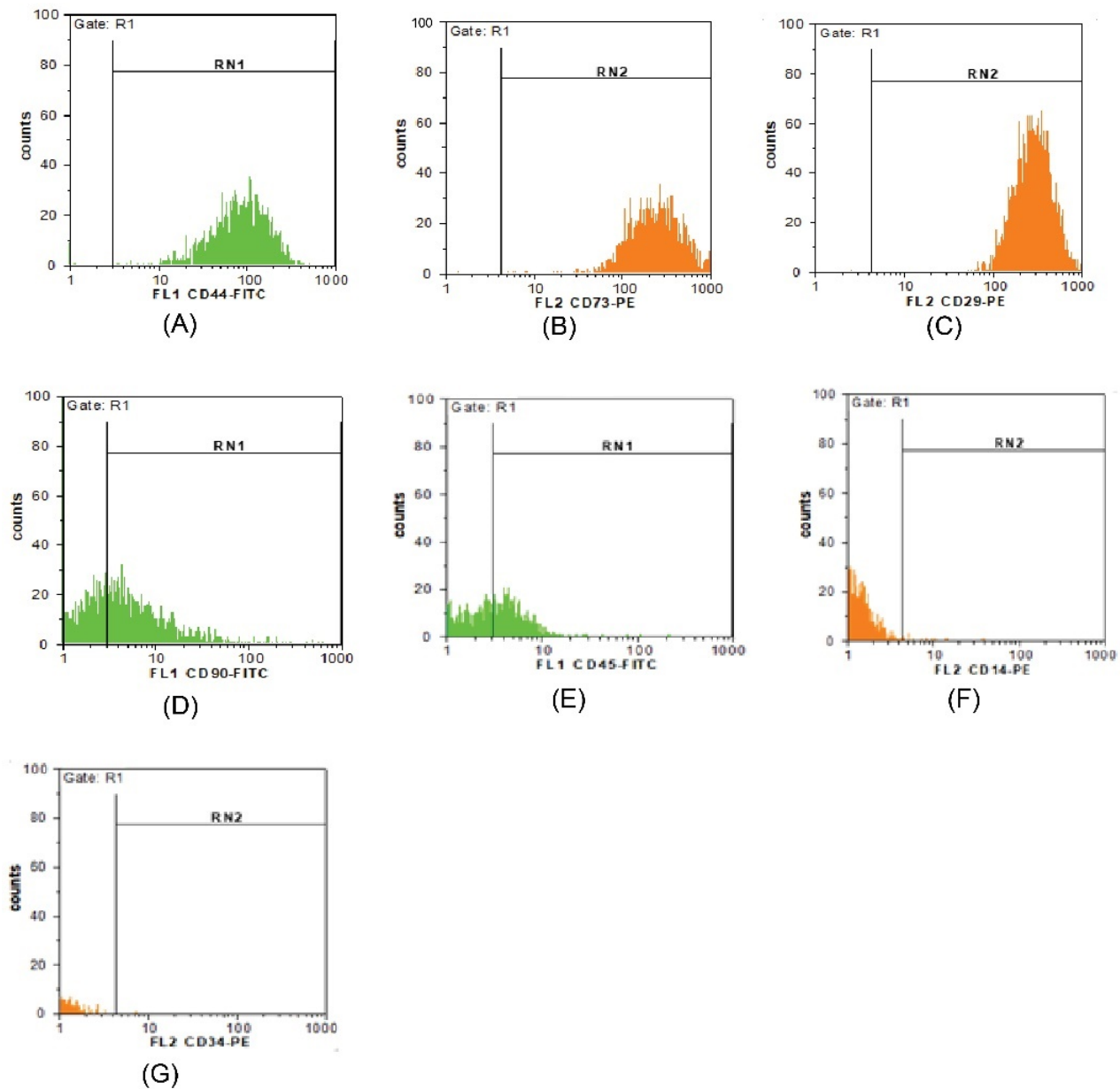
**Figure 2.** Human Amniotic Membrane Derived Mesenchymal Stem Cells on days 9-10 With Enzymatic Isolation (10X).



**Figure 3.** Percentage of Viability and Apoptosis in Human Amniotic Membrane Derived Mesenchymal Stem Cells Before Transplantation.

### Flow Cytometry of hAMSCs

To confirm the origin of mesenchymal cells, we used flow cytometry analysis to assess surface markers and identify the phenotype of the stem cells isolated from the amniotic membrane. The results showed that the isolated mesenchymal cells expressed each of the positive markers of mesenchymal cells at a high level and expressed the negative markers at a very low level. In sum, the above results prove the mesenchymal nature of cells isolated from the amniotic membrane (Figure 4). These cells express the markers CD44 (99.43%) and CD73 (99.72%). The expression rate of these two markers in total was



**Figure 4.** Expression of Positive Markers CD44 (A), CD73 (B), CD29 (C), and CD90 (D), and No (or Low) Expression of Markers CD45 (E), CD14 (F), and CD34 (G) in hAMSCs.

99.38%. In addition, these cells express the markers CD29 (44.64%) and CD90 (99.78%). The expression rate of these two markers in total was 44.56%; also, CD34 marker (0.07%) expression was 0.04%. These cells express the markers CD14 (0.78%) and CD 45 (18.95%). The expression rate of these two markers in total was 0.41%.

#### **Differentiation Potential of hAMSCs**

The results showed that these cells could differentiate into fat and bone cells (Figure 5). In general, the above results prove the mesenchymal nature of cells isolated from the amniotic membrane.

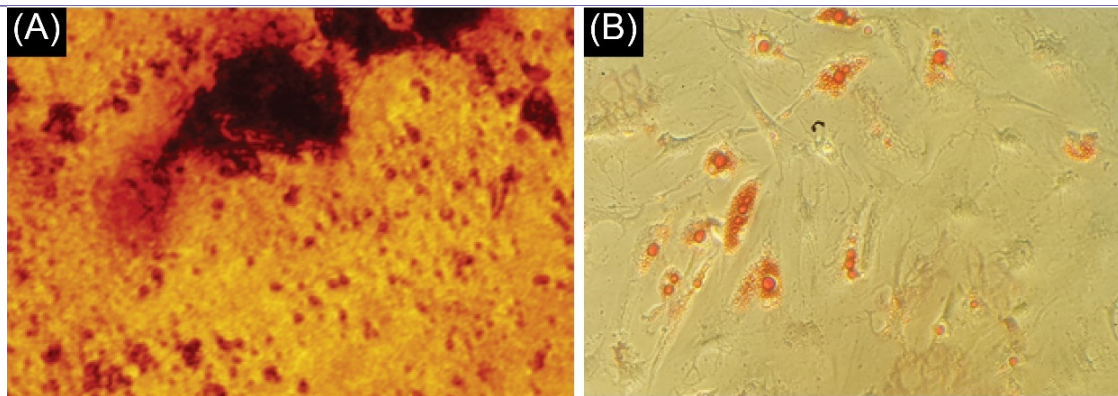
#### **Evaluation of Wound Closure**

Gross observation revealed that wound healing had a

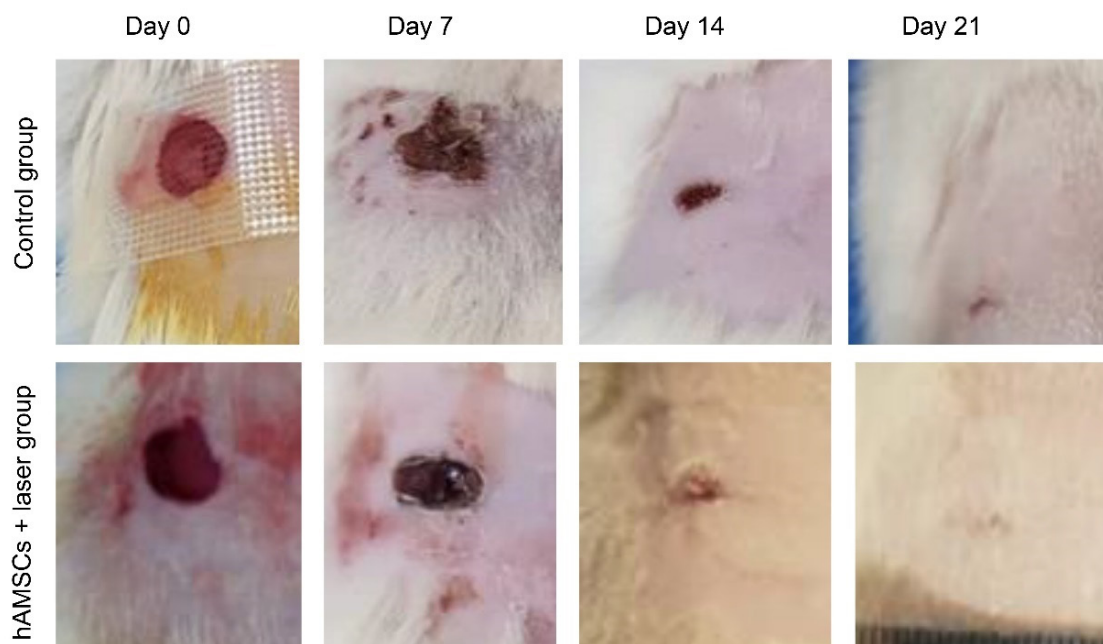
faster rate in the amniotic MSC group irradiated with a diode laser than in the amniotic MSC group (Figure 6).

#### **Histopathological Evaluation of the Wound**

Hematoxylin and eosin (H&E) staining revealed that the neutrophil count peaked on day 3 after ulceration and then decreased in both groups. Therefore, it can be concluded that both groups do not stimulate the immune system and that they cause an inflammatory reaction and increase the number of neutrophils at the wound site. In addition, the number of macrophages on day 7 in the irradiated cell group was lower than that in the cell group. On day 21, although the number of macrophages decreased in both groups, this number was significantly lower in the irradiated cell group than that in



**Figure 5.** Differentiation of hAMSCs Into Osteocytes (A) and Adipocytes (B).



**Figure 6.** Wound Closure in the Control Group and in the hAMSCs Injection+Laser Group on Days 0, 7, 14, and 21.

the cell group. Epithelial formation was observed on day 7 and increased until day 14. On days 7, 14 and 21, the percentage of epithelial formation in the irradiated cell group was significantly higher than that in the cell group, so that on day 21, the epithelium in this group completely covered the wound surface while in the control group the wound surface was still not completely covered. On day 7, angiogenesis was significantly lower in the irradiated cell group than that in the cell group. Therefore, it can be said that in the irradiated group, the wound needs nutrient flow, and in fact, the formation of new blood vessels is reduced (Figure 7).

Masson trichrome staining showed deposition of the mature collagen matrix in both groups but a more organized and more intense degree of collagen deposition in the diode laser irradiated hAMSCs group (Figure 7).

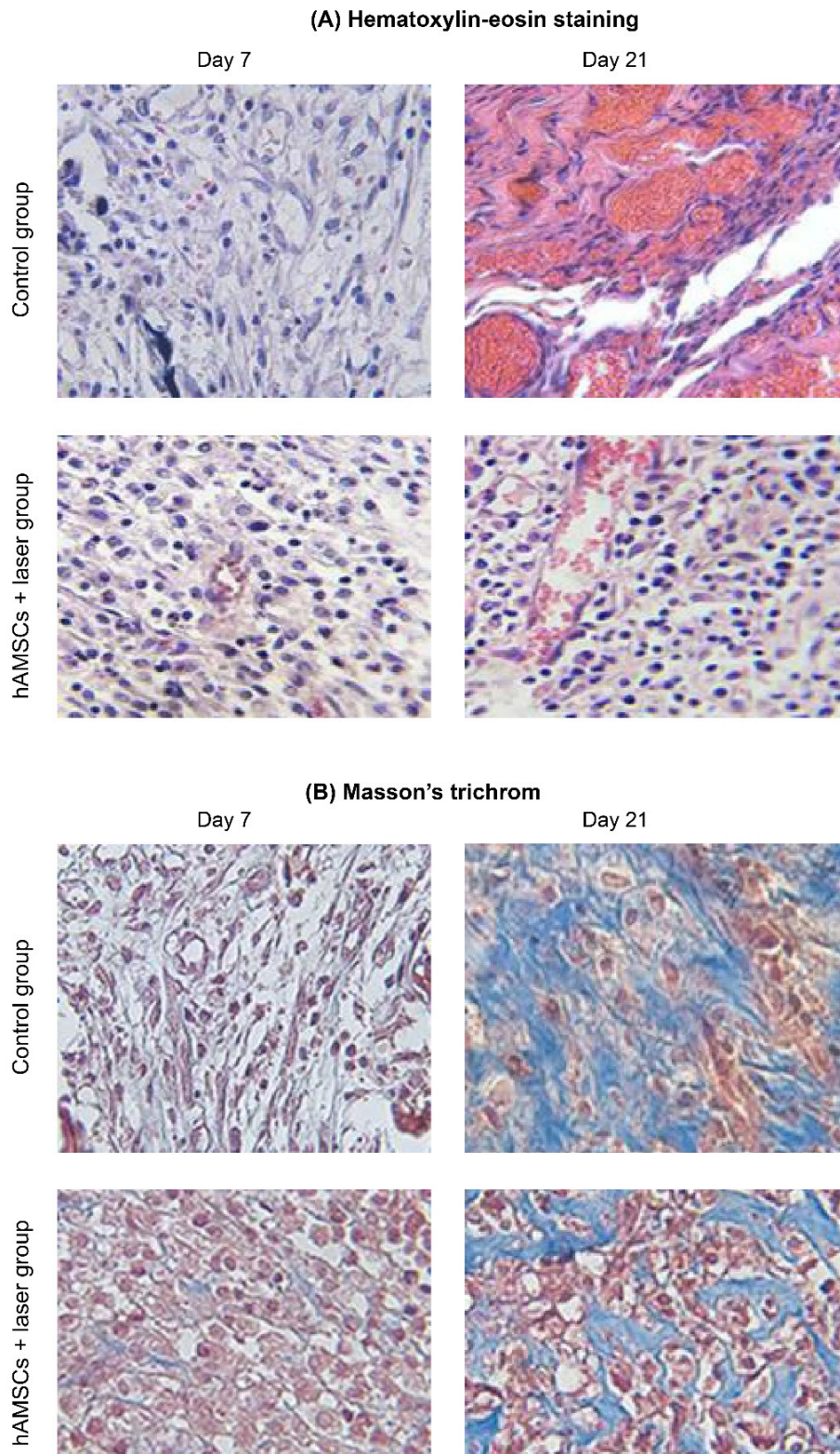
#### ***The Level of Changes in the Thickness and Density of the Epidermal Layer***

Ultrasound imaging results showed that the thickness and

density of the epidermis in the wound area were higher in the hAMSCs+laser injection group on days 7, 14 and 21 than in the control group. Results in both groups showed a significant difference between epidermal thickness on days 7, 14 and 21; however, there was no significant difference between two groups on day 7, while the thickness and density of wound epidermis on days 14 and 21 were significantly different between the two groups ( $P < 0.001$ ), and they were significantly higher in the hAMSCs+laser injection group than in the control group, which indicated faster wound healing in the hAMSCs+laser injection group than in the control group (Figure 8).

#### ***The Level of Changes in the Thickness and Density of the Dermis Layer***

According to the ultrasound imaging results, the thickness and density of the dermis in the wound area were higher in the hAMSCs injection+laser group on days 7, 14 and 21 than in the control group. Examination

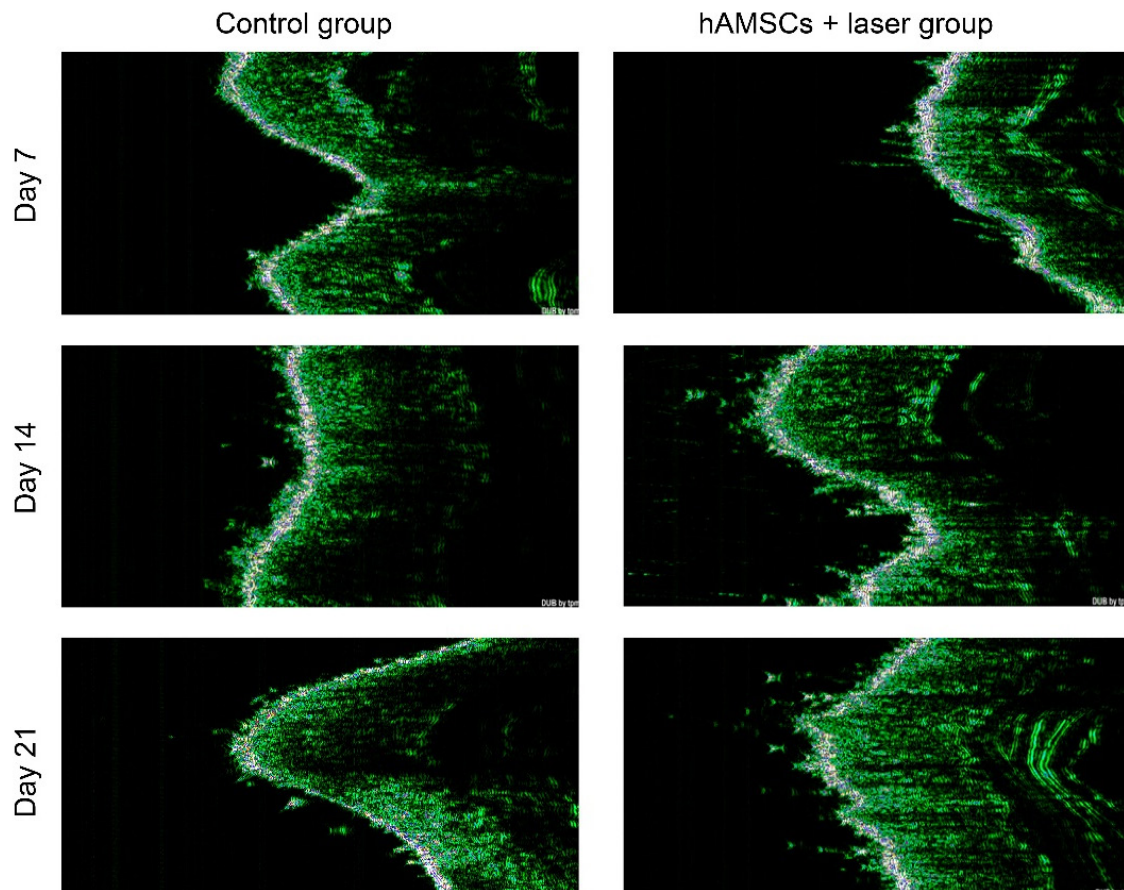


**Figure 7.** Hematoxylin-Eosin Staining in the Control and hAMSCs+Laser Groups on Days 7 and 21 (A); Masson's Trichrome Staining in the Control and hAMSCs Injection+Laser Groups on Days 7 and 21 (B).

of dermis thickness and density between the control and hAMSCs injection+laser groups on different days showed that there was no significant difference between the two groups on day 7, while dermis thickness and density on days 14 and 21 showed a significant difference

between the two groups, and they were higher in the hAMSCs injection+laser group than in the control group. This indicated faster wound healing in the hAMSCs injection+laser group than in the control group.

According to the results obtained, the dermis thickness



**Figure 8.** Ultrasound Imaging of the Wound Area in the Control and hAMSCs Injection Groups on days 7 and 21.

of the wound area on day 7, a significant increase was observed in the group receiving hAMSCs+laser compared to the control group ( $P=0.000$ ,  $\alpha=0.05$ ). Also, the dermis thickness of the wound area on day 14 showed a significant increase in the group receiving hAMSCs+laser compared to the control group ( $P=0.002$ ,  $\alpha=0.05$ ). In this regard, the dermis thickness of the wound area on day 21 also had a significant increase in the group receiving hAMSCs+laser compared to the control group ( $P=0.000$ ,  $\alpha=0.05$ ) (Figure 8).

#### **The Level of Elasticity Changes in the Wound Area**

According to the results of the cutometer, the elasticity in the wound area was higher in the hAMSCs injection+laser group on days 7, 14 and 21 than in the control group. In the study of elasticity changes in rats in the control group and in the hAMSCs injection+laser group, a significant difference was observed between wound elasticity on days 7, 14 and 21. Wound elasticity between the control group and the hAMSCs injection+laser group on different days showed that there was no significant difference between the two groups on day 7, while wound elasticity on days 14 and 21 was significantly different between the two groups, and it was significantly higher in the hAMSCs injection+laser group than in the control group. This issue indicated faster wound healing in the hAMSCs

injection+laser group than in the control group.

Elasticity (R2) of the wound area was on day 7 ( $P=0.008$ ,  $\alpha=0.05$ ), day 14 ( $P=0.005$ ,  $\alpha=0.05$ ), and day 21 ( $P=0.002$ ,  $\alpha=0.05$ ); a significant increase was observed in the group receiving hAMSCs+laser compared to the control group.

Elasticity (R5) of the wound area was on day 7 ( $P=0.000$ ,  $\alpha=0.05$ ), day 14 ( $P=0.000$ ,  $\alpha=0.05$ ), and day 21 ( $P=0.000$ ,  $\alpha=0.05$ ); a significant increase was observed in the group receiving hAMSCs+laser compared to the control group.

Elasticity (R7) of the wound area was on day 7 ( $P=0.000$ ,  $\alpha=0.05$ ), day 14 ( $P=0.000$ ,  $\alpha=0.05$ ), and day 21 ( $P=0.000$ ,  $\alpha=0.05$ ); a significant increase was observed in the group receiving hAMSCs+laser compared to the control group.

#### **Discussion**

This study showed that epithelial formation was on day 7 and increased until day 14. On days 7, 14 and 21, the percentage of epithelial formation in the irradiated cell group was significantly higher than that in the cell group, so that on day 21, the epithelium in this group completely covered the wound surface while in the group control the wound surface was still not completely covered. In terms of wound angiogenesis, on day 7, the irradiated cells were significantly lower than the cell groups. In addition, the formation of collagen in the cellular hydrogel group can confirm the effectiveness of amniotic MSCs in collagen

production and thus accelerate the wound healing process. In some studies, LLLT as an alternative or adjunctive method is used for different types of wounds when there is no definitive approach.<sup>15</sup> Several studies have been conducted to develop LLLT for chronic wounds, and several studies have addressed diabetic ulcers. Necrotic tissue due to hyperglycemia causes the most complications such as skin wounds and deep and superficial wounds caused by venous and arterial blockage.<sup>16</sup>

One of the problems of using LLLT is using the optimal dose of exposure.<sup>17</sup> Much research has been done on tissues, and it has been proven that the depth of laser penetration varies in different tissues; for example, oral mucosa does not absorb light well, and bone and skin are almost similar to mucosa, while muscles absorb the most light. LLLT can be used in such classifications as abrasions knife cuts, para-scrotal wet eczema, necrotic wounds, and inflammation.<sup>18</sup> Also, some studies have mentioned that LLLT has analgesic, vasodilation and anti-inflammatory properties.<sup>19,20</sup>

In a study, Werber and Martin examined the therapeutic effect of amniotic fluid transplantation on chronic wounds and found that a total of 18 wounds (90%) healed over a 12-week period, and none of the wounds led to amputation<sup>21</sup>; therefore, in their evaluation, which is consistent with the present study, the effectiveness of this method was cleared. Also, Zafari et al, in a study, evaluated the therapeutic effect of stem cell transplantation on the healing of chronic wounds in diabetic patients and observed that in terms of the wound surface and percentage of healing, the largest differences were in the cell group and the control group, respectively ( $P < 0.05$  and  $P < 0.01$  respectively). Immunohistochemistry also showed the survival and activity of CD93 stem cells at the wound site up to 28 days after transplantation. At the end of their study, they stated that CD 93 stem cell transplantation significantly accelerated the healing process of diabetic wounds.<sup>22</sup> In another study, Shojaei et al evaluated the therapeutic effect of LLLT and observed that LLLT combined with routine treatment (case group) compared to routine treatment (control group) had a significant effect on reducing the wound stage and wound size,<sup>23</sup> which is consistent with our assessment. Also in a review study, Posten et al found that although some researchers have reported an increase in cell proliferation and collagen production using HeNe and GaAs lasers, none of these articles have reported the mechanism of action of LLLT.<sup>24</sup> The novelty and difference in our study were that we applied LLLT to the hAMSCs and showed that it had a more beneficial effect on wound healing than using the hAMSCs alone. However, there is a need for more studies with a larger sample size to evaluate the mechanism of LLLT action in wound healing.

## Conclusion

A low-power laser along with MSCs can be effective

in improving chronic wound conditions in the animal model; in comparison with hAMSCs alone, irradiated hAMSCs accelerate the wound healing process and increase the thickness and elasticity of the skin.

## Conflict of Interests

The authors declare that they have no conflict of interest.

## Ethical Considerations

This study was approved by the Medical Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (ethic No: IR.SBMU.MSP.1398.508).

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