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A randomized, double-blind, phase I clinical trial of fetal cell-based skin substitutes on healing of donor sites in burn patients

Mahnoush Momeni [a,1], Nasrin Fallah [b,1], Amir Bajouri [b,1], Tooran Bagheri [a], Zahra Orouji [b], Parisa Pahlevanpour [a], Saeed Shafieyan [b], Niloofar Sodeifi [c], Ahad Alizadeh [d], Nasser Aghdami [b,*], Mohammad Javad Fatemi [a,*]

[a] Burn Research Center, Iran University of Medical Sciences, Tehran, Iran
[b] Department of Regenerative Medicine, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
[c] Department of Pathology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
[d] Department of Epidemiology and Reproductive Health, Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

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ABSTRACT

Background: Due to limited graft donor sites in extensive burns, re-harvesting of a single donor area is very common. Given the importance of fetal fibroblasts in accelerating fetal wound healing, fetal cell-based skin substitutes have emerged as a novel therapeutic modality for regenerating damaged skin. In this trial, we aimed to evaluate the safety, feasibility and potential efficacy of application of amniotic membranes seeded with fetal fibroblasts for accelerating donor sites healing in burn patients.

Methods: In this randomized, double-blind, phase I clinical trial, 10 patients with total burn surface area of 10-55% were enrolled. Three equal parts (10 × 10 cm) were selected in donor site of each patient and covered by Vaseline gauze (control group), amniotic membrane (AM group), or amniotic membrane seeded with fetal fibroblasts (AM-F group). Adverse events, pain intensity scores, and wound sizes were recorded on days 4, 8, 11, 14, and 20 post-treatment. Also, histological assessments were done on days 0 and 14 after the surgery.

Results: All patients underwent surgery, and no adverse events occurred during the procedure and follow-up period. Significantly lower pain intensity and higher healing rates were observed in AM-F and AM groups compared to the control group. Moreover, mean complete re-epithelializatin in AM-F and AM groups were 10.1±2.4 and 11.3±2.9 days, showing that the healing process was significantly accelerated compared to the control group with mean closure time of 14.8±1.6 days. Histological assessment showed lower inflammatory cells infiltration in AM-F and AM groups compared to control group.

Conclusions: This study indicated the safety of transplantation of amniotic membrane seeded with fetal fibroblasts for treatment of donor sites in burn patients; however, preliminary

* *Corresponding authors.*
E-mail addresses: nasser.aghdami@royaninstitute.org (N. Aghdami), mjfatemi41@gmail.com (M.J. Fatemi).
[1] These authors contributed equally to this article.
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assessments showed no benefits for this therapeutic modality over amniotic membrane alone. Thus, to draw accurate conclusions, further trials in larger populations should be conducted. Level of Evidence: This study is assigned as level I.

1. Introduction

Despite remarkable progress in burn management, these injuries are a leading cause of mortality and morbidity, especially in low-income countries [1,2]. Currently, early excision and autografting are the standard management of deep partial- and full-thickness burns [3]. Due to limited graft donor sites in extensive burns, re-harvesting of the same area is very common [4]. Severe burns have local and systemic devastating effects that impair the normal healing process even in the donor area [5]. Hence, accelerating donor area healing would lead to more frequent graft harvest, more effective treatment of the burn patients, shorter hospital stay, and less pain and scarring [4].

The ideal dressing should speed up re-epithelialization, prevent infection, and be painless, adjustable for different sites, easy to use, and cost-effective. To date, several covering materials have been introduced for burn and donor sites treatment including synthetic membranes, autologous or allogenic biological approaches, and more recently, stem cells-based strategies and tissue engineering [6–8].

Amniotic membrane, as the innermost layer of the fetal membranes, is known as a traditional bio-scaffold since its introduction in 1910 [9]. Several studies have reported the anti-inflammatory, anti-microbial, wound-healing, and anti-scar properties of amniotic membrane which are due to its ability to excrete numerous growth factors and cytokines that induce tissue regeneration [10]. Recent studies revealed that concurrent application of bio-scaffolds and stem cells could result in more efficient cell engraftment and host-cell infiltration [10,11].

Considering the fact that fetal skin wounds heal rapidly and regenerate faster compared to adults, it is presumed that fetal cells hold the key for tissue regeneration [12,13]. Several studies have shown that compared to adult fibroblasts, fetal fibroblasts possess greater proliferative and migratory capacity, larger amounts of extracellular matrix deposition and different growth factors [14–16]. Given the importance of fetal fibroblasts in accelerating fetal wound healing, researchers have tried to mimic the fetal wound healing conditions by using fetal cell-based skin substitutes [12,15]. It has been shown that fetal cells migrate into the wound bed and provide growth factors and cytokines necessary for efficient tissue regeneration [17]. Furthermore, contrary to the allogeneic cells, the fetal fibroblasts have high expansion capacity under simple culture conditions and do not evoke immunological responses in the recipient site [18,19].

To date, several studies have demonstrated the safety and efficacy of cell-based skin substitutes in healing full-thickness wounds [20–22]. In this study, we aimed to evaluate the safety, feasibility and potential efficacy of application of amniotic membrane seeded with fetal fibroblasts for accelerating donor sites healing in burn patients and compare the results with those obtained following application of commonly used amniotic membrane or Vaseline gauze.

2. Materials and methods

2.1. Patients and study design

In this randomized, double-blind, phase I clinical trial, we evaluated the safety, feasibility and potential efficacy of application of amniotic membrane seeded with fetal fibroblasts in comparison with amniotic membrane and Vaseline gauze, for donor sites healing in burn patients. The sample size was estimated using package long power according to "Sample Size Calculations for Longitudinal Data" by R software [23]. A total of 10 patients who were admitted to the burn department of Motahari Hospital in Tehran, Iran, from 2014 to 2016, were enrolled in the present trial. Subjects were of both sexes, aged between 12 and 60 years, had total burn surface area (TBSA) of 10–55%, and referred to the hospital within the first 24 h after burn trauma and they were candidates for autograft. Patients with a history of underlying chronic diseases, electrical or chemical burn, inhalation injury, organ transplantation or blood transfusion, cancer, hepatitis B or C or HIV and those who were pregnant or lactating, were not included. The treatment process was explained to each eligible patient and he/she signed the informed consent before participation in this trial. The study was approved by the ethics committee of Iran University of Medical Sciences and registered at www.irct.ir with registration No. IRCT201302218177N6.

After admission, patients received appropriate resuscitation depending on burn severity. After excision of burn sites and the necrotic tissues, a standardized split-thickness skin graft of 0.4 mm thickness was harvested from the thigh of the patients using a dermatome (Acculan 3Ti Dermatom, FA. Aesculap AG, Tuttlingen, Germany). In the donor site of each patient, we selected three wounds of equal size (10 × 10 cm) with 5 cm intervals. Then, the wounds were randomly covered by gauze impregnated with Vaseline (control group), amniotic membrane (AM group), and or amniotic membrane seeded with fetal fibroblasts (AM-F group), according to simple random allocation method. We gently put the amniotic membrane with or without cells, on the donor sites and then covered the sites with Vaseline gauze. The dressings remained intact for four days. Patients received visits on days 4, 8, 11, 14 and 20 after the surgery. Vaseline dressings in all groups were changed in each follow-up visit and wound sites were photographed. A schematic presentation of the study design is shown in Fig. 1.

2.2. Primary and secondary endpoints

Primary endpoints included frequency, type, and severity of adverse events (AEs) in donor sites following treatment. In

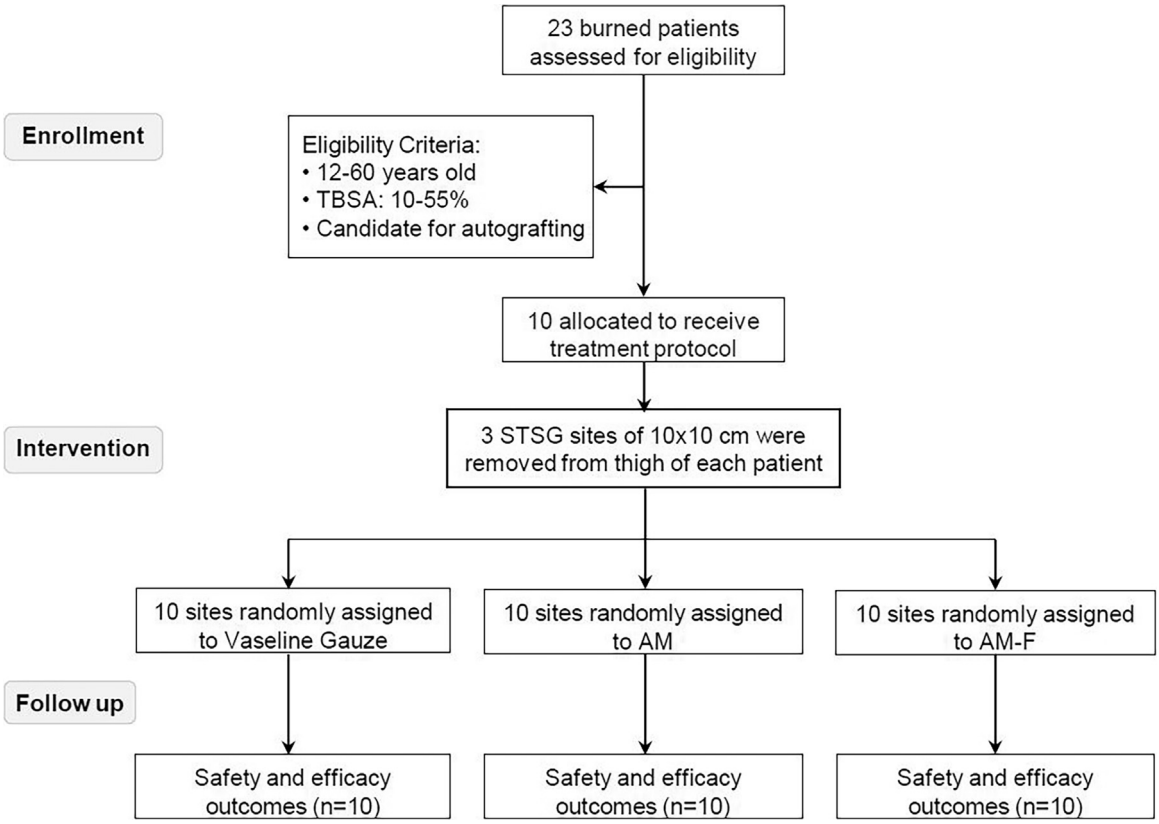

Fig. 1 – A schematic presentation of the trial design.
TBSA: total body surface area, STSG: split-thickness skin graft, AM: amniotic membrane, AM-F: amniotic membrane seeded with fetal fibroblasts.

this regard, we recorded AEs during follow-up visits based on Common Terminology Criteria for Adverse Events 2010 (CTCAE [24]). Also, patients in each group were asked to rate their pain severity (from 0 to 10) using a numerical pain intensity scale (NPIS) which a score of 10 indicated the most severe pain. An expert burn surgeon evaluated wound infection in donor sites.

Secondary endpoints consisted of re-epithelialization rates evaluated in each visit. Three independent expert burn surgeons who were blinded to the dressing types, evaluated the healing process using wound photographies. The re-epithelialization rate was calculated according to improvements in wound surface area compared to the original wound area. The amount of wound closure was measured using the following equation:

$$(\text{original wound area} - \text{actual wound area})/\text{original wound area} \times 100\%$$

Furthermore, 14 days after the treatment, a 2-mm punch biopsy was taken from the center of each treatment site for histological assessments.

2.3. *Histological assessment*

For histological studies, glass-slide biopsy specimens were stained by Hematoxylin and Eosin (H&E) and Masson's trichrome. The slides were evaluated in terms of epidermal and dermal structures, acute and chronic inflammatory cells infiltration and fibrosis under an Olympus light microscope model CKX-41 and Nikon Eclipse E200.

The epidermis was evaluated for remaining ulcers, the degree of re-epithelialization, and the new epithelium thickness. Furthermore, the dermis was evaluated for degree of edema using H&E staining and the percentage of dermal fibrosis using Masson's trichrome-stained slides (fibrosis of 0–100% of whole dermal volume) compared to non-damaged skin.

The level and composition of inflammatory cells infiltrated in epidermis and dermis were categorized as follows: acute (polymorphonuclear cells and eosinophils), phagocytic chronic (histiocytes) and non-phagocytic chronic (lymphocytes and plasma cells). Inflammatory cells were counted separately in at least 20 high power fields (hpf=400×) and the mean count was reported using the following scoring system: 0: no inflammatory cell; 1: Inflammatory cell count of 1–5 per hpf; 2: Inflammatory cell count of 6–25 per hpf; 3: Inflammatory cell count of 26–50 per hpf; 4: Inflammatory cell count of 51–75 per hpf; 5: Inflammatory cell count of 76–100 per hpf and 6: Inflammatory cell count of >100 per hpf.

2.4. *Preparation of cells and human amniotic membrane (HAM)*

All the biologic dressings were prepared in Royan Institute and transferred to the hospital.

2.5. Cell isolation and cultivation

Fetal skin specimen from normal and non-macerated fetuses of 11–14 weeks old, were collected after spontaneous pregnancy termination. None of the donors had human immunodeficiency virus, hepatitis B or C, or syphilis. They all signed an informed consent before donating the fetus.

Fetal fibroblasts were isolated and cultivated according to our previous study [25]. Briefly, after washing the skin biopsy in Hank's buffered salt solution (HBSS, Gibco, USA) containing 100U/ml penicillin and 100μg/ml streptomycin (Gibco, USA), the dispase solution (Gibco, USA, 1.2U/ml) was applied for isolating epidermis from the dermis layers at 4°C, overnight. The dermis layer was cut into small pieces and incubated with 0.1% collagenase I enzyme (Sigma, USA) for 3h at 37°C. After inactivating collagenase I enzyme by DMEM/F12+10% fetal bovine serum medium (Hyclone, USA), the cells' pellet was filtered through 70-μm Falcon™ Cell Strainers (BD, USA). Then, it was centrifuged at 1500rpm for 5min. Finally, the isolated fibroblasts were cultured by seeding 3×10^5 cells in one well of a 6-well cell culture plate in DMEM-F12 (Gibco, USA) containing 10% fetal bovine serum (Hyclone, USA), L-glutamine (Gibco, USA), 100U/ml penicillin and 100μg/ml streptomycin at 37°C, with 5% CO_2 and 95% humidity. The culture medium was changed every three days. Fetal fibroblasts were collected and characterized at passage 3, and then seeded on HAM.

2.6. Cell characterization

2.6.1. Morphological analysis

In-vitro expanded fetal fibroblasts were evaluated under an inverted microscope Olympus CKX-41, at passage 3.

2.6.2. Flow cytometry

Fetal fibroblasts were identified based on the presence of CD73, CD90, CD29, CD26 and vimentin and absence of the hematopoietic lineages' markers including CD34 and CD45 [26]. For this purpose, at passage 3, fetal fibroblasts were re-suspended in a 3% bovine serum albumin solution (BSA; Sigma-Aldrich, USA) for 20min at room temperature. The cells were washed with phosphate-buffered saline (PBS; Gibco, USA). Then, fetal fibroblasts were incubated with mouse anti-human antibodies for vimentin-phycoerythrin, CD73, CD90, CD29, CD26, and CD45, and CD34 (BD Biosciences, USA). The cells were centrifuged and analyzed by a FACsCalibur Flow Cytometer (BD Biosciences, USA) using the CELL Quest software version 3.3.

2.7. HAM preparation

HAMs were prepared in HAMs bank which is a part of the public cord blood bank of Royan Institute, Tehran, Iran. At this stage, $10\times10\,cm^2$ pieces of HAMs were incubated with trypsin–EDTA (ethylene-diamine-tetraacetic acid) solution at 37°C for 5min. HAMs epithelial cells were gently removed by a cell scraper. Then, the HAMs were washed three times with PBS.

2.8. Cell-based substitutes preparation

Human fetal fibroblasts were seeded on pieces of the acellular amniotic membrane at a density of $5\times10^5/cm^2$ and incubated for 7 days. Afterward, the skin substitute derived from amniotic membranes was cut into $2\times10\,cm^2$ pieces and transplanted in the excised burned area.

2.9. Statistical analysis

The data were analyzed using SPSS software version 21.0 (Chicago, IL, USA). Descriptive statistics were presented as mean±SD for continuous variables and percentages for categorical variables. Two factors (groups and time) were compared by repeated analysis of variance and analysis of covariance (ANCOVA), followed by the Bonferroni and least significant difference *post-hoc* tests. All statistical tests were two-sided and a p-value of less than 0.05 was considered significant.

3. Results

3.1. Cells and skin substitutes' characteristics

Microscopic observation of passage-3 fetal fibroblasts revealed a monolayer of spindle-shaped cells. The flow cytometry analysis showed that CD73, CD90, CD29, CD26, and vimentin markers were highly expressed by fetal fibroblasts. The microscopic evaluation of fetal fibroblasts seeded on HAM showed normal morphology (Fig. 2).

3.2. Patients

From a total of 10 patients with acute burn who were enrolled in the present study, 9 patients were male (90%) and the mean age of the patients was 29±10years. All patients had thermal burn and the mean TBSA percentage of the patients was 30 ±17%. The patients underwent different treatments and then received visits on days 4, 8, 11, and 14, followed by every-3-day visits until the end of the hospitalization period. Average hospitalization period was 23±5 days for our patients. Baseline characteristics of the patients are listed in Table 1.

3.3. Safety and tolerability

All the patients survived until the end of the hospitalization time. Patients were discharged after complete treatment with no severe AEs during the treatment period. On average, the donor sites healed after 12.1±3.1 days while no infection or major AEs were observed in the wounds of all three groups. Pain intensity assessments showed that on day 4, patients experienced pain severity of 5.9, 5.4, and 6.5 in AM-F, AM and control group, respectively. On day 8, severity scores were 2.4, 3.1, and 5.3 for AM-F, AM, and control group, respectively ($p<0.05$). Pain intensity scores on days 11 and 14 were significantly reduced in AM-F and AM groups compared to the control group (Fig. 3).

3.4. Efficacy outcomes

We observed normal re-epithelialization in all treatment sites during the study. The sizes of the treatment sites were approximately $100\pm6\,cm^2$ and did not vary significantly

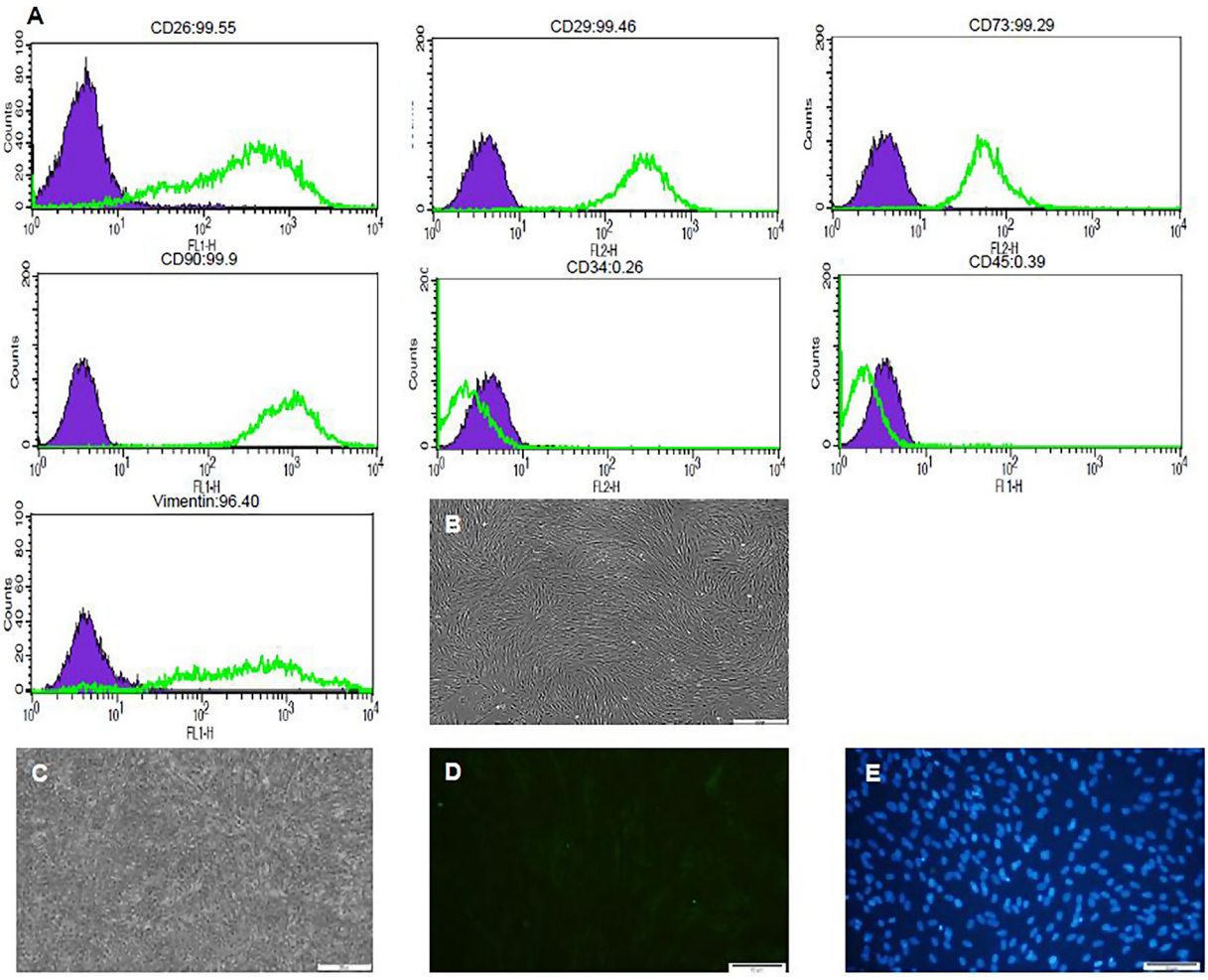

Fig. 2 – (A) Flow cytometry diagrams. Fetal fibroblasts expressed CD26, CD29, CD73, CD90 and vimentin cytoplasmic markers but not CD34 and CD45 markers. The peak levels of black and green histograms are isotope control and markers expression. (B) Morphology of fetal fibroblasts at passage 3. (C) Appearance of fetal fibroblast cells cultured on AM by day 7 after seeding. (D) Immunocytochemistry of fetal fibroblast cultured on AM were positive for vimentin. (E) Cell nuclei were stained using DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

among the three groups. On day 4 post-surgery, re-epithelialization measurements showed that the wound size in AM-F and AM group were reduced by 40.7% and 37.0%, respectively which were significantly greater than that of the control group (24.9%). After 8 days, the wounds in AM-F, AM and control group showed 74.5, 74.5 and 34.5% closure, respectively ($p < 0.05$). On day 11, AM-F and AM had wound closures of 94.0 and 95.5%, respectively which were markedly greater than

Table 1 – Patient characteristics at the time of enrollment.

Patient number	Age (years)	Sex (F/M)	TBSA (%)	Hospital stay (days)	Concurrent disease	Educational level	Employment
1	39	M	40	19	–	Completed primary school	Self-employment
2	24	M	6	24	–	Completed high school	Self-employment
3	24	M	29	31	–	Completed high school	Soldier
4	44	M	47	27	–	Completed high school	Self-employment
5	25	M	55	25	–	Completed high school	Self-employment
6	36	M	10	25	+	Completed high school	Part-time
7	46	M	45	30	–	Completed primary school	Part-time
8	23	M	42	18	–	Completed high school	Part-time
9	13	F	15	17	–	Primary school	Student
10	21	M	15	14	–	Completed high school	Part-time

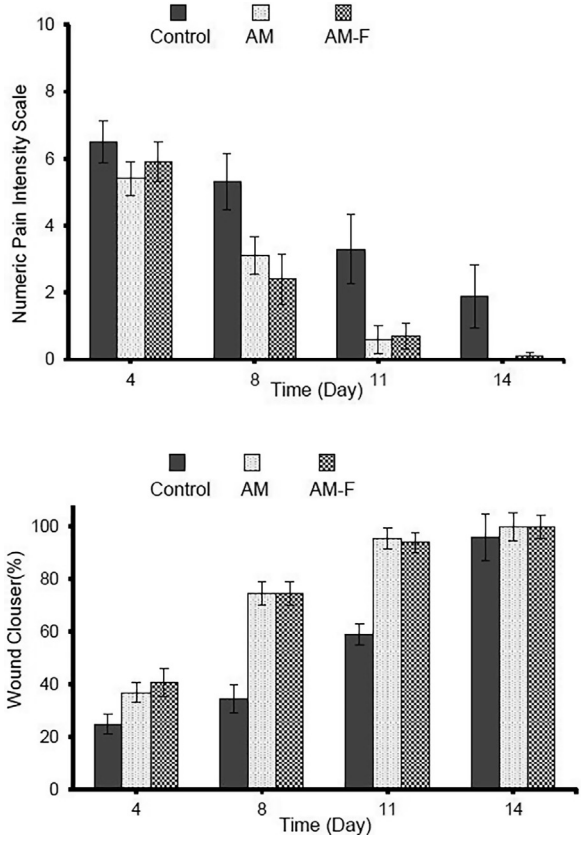

Fig. 3 – (A) Pain intensity scores in treatment groups over time. (B) Re-epithelialization rate in treatment groups over time.
AM: amniotic membrane, AM-F: amniotic membrane seeded with fetal fibroblasts.

that of control group (59.0%; $p < 0.05$). Wound closure measurements during post-surgery visits are represented in Figs. 3 and 4. Wounds of AM-F and AM groups were almost closed, 10.1 ±2.4 and 11.3±2.9 days after initiation of the treatment, while in control group, it took 14.8±1.6 days for wounds to close (Figs. 3 and 4).

3.5. *Histopathology*

Histological evaluation of the biopsy specimens revealed that the wound of all three groups were almost completely healed. Findings revealed gradual progression in partial epidermal re-epithelialization but with decreased thickness. Moreover, dermis showed edema, mild-to-moderate acute and chronic inflammatory cells infiltration and fibrosis as well as partial dermal appendages' destruction. Two weeks after transplantation, mean scores of PMN infiltration (acute inflammation cells) of all groups were 2. Moreover, the mean score of non-phagocytic cells in control group was 2.5, while in AM and AM-F groups, the mean score of non-phagocytic cells was 1.5. Assessment of the phagocytic cells infiltration in different groups showed inflammatory cells infiltration scores of 1.7 in AM-F group versus 2.6 in AM and control groups, respectively. Re-epithelialization showed accelerated wound regeneration in AM-F and AM groups (mean score 1.6 for both groups) compared to control group (mean score 1). Furthermore, mean dermal edema in control, AM, and AM-F groups were 2, 2.2, and 1.8, respectively. Regarding fibrosis percentages, evaluation of Masson's trichrome-stained specimen showed that mean fibrosis percentages in control, AM, and AM-F groups were 63.5, 59.6, and 64.8%, respectively. Histological findings of patient number 4 are presented in Fig. 5.

4. Discussion

The results of this randomized clinical trial demonstrated that transplantation of amniotic membrane and fetal fibroblast into donor sites in burn patients, was safe and it was well-tolerated. Furthermore, patients experienced lower pain intensity in the treatment sites during the healing period in comparison with the control sites. Efficacy outcomes revealed a faster healing time in the sites covered by fetal skin substitutes compared to the placebo-treated sites. In the clinical setting, donor-site healing time is critical in cases with extensive burn. In our study, AM-F and AM-treated wounds were averagely closed 10.1±2.4 and 11.3±2.9 days after initiation of the treatment, while in the control group, it took 14.8±1.6 days. It enabled the physician to re-harvest the same donor area sooner, which was also associated with less pain, infection, and inflammation. In this trial, the pain was considered as a primary endpoint and a visual pain analog scale was used for its quantification. We found that pain tolerance in patients who received amniotic membrane alone or amniotic membrane seeded with fetal cells was significantly improved. The majority of the patients tolerated the fetal-based skin substitutes very well. Consistently, previous studies indicated less pain intensity at donor sites following application of amniotic membrane [27].

Recently, a randomized clinical trial that evaluated the effect of various dressings on healing time of donor sites after split-skin harvesting, showed a mean healing time of 27.9 (18-33) days when covered with Vaseline gauze [28]. Also, a study that reviewed donor-site healing time in 33 studies, demonstrated that donor sites-under optimal conditions-were healed within 7–21 days [29]. Our study revealed that the donor-sites wounds covered with Vaseline gauze were averagely closed after 14.8±1.6 days.

The studies that evaluated the effect of amniotic membrane on donor-site healing time, revealed a mean closure time of 12–18 days [27,30]. Loeffelbein demonstrated that 93.3% of the wounds covered with amniotic membrane were completely epithelialized on day 12, whereas 86.7% of the wounds were epithelialized in the control groups covered by polyurethane scaffolds [30]. Besides, we observed that the average wound closure times in AM-F and AM groups were 10.1±2.4 and 11.3 ±2.9 days, respectively, which showed a significant acceleration of wound closure process compared to the control group. Our study showed no difference in donor-site healing time between the amniotic membrane alone-treated group and the group treated with amniotic membrane seeded with fetal fibroblasts. This is in contrast to our previous study that demonstrated faster healing rates for rats' large full-thickness burn wounds treated with amniotic membrane seeded with fetal fibroblasts compared to the amniotic membrane alone [22].

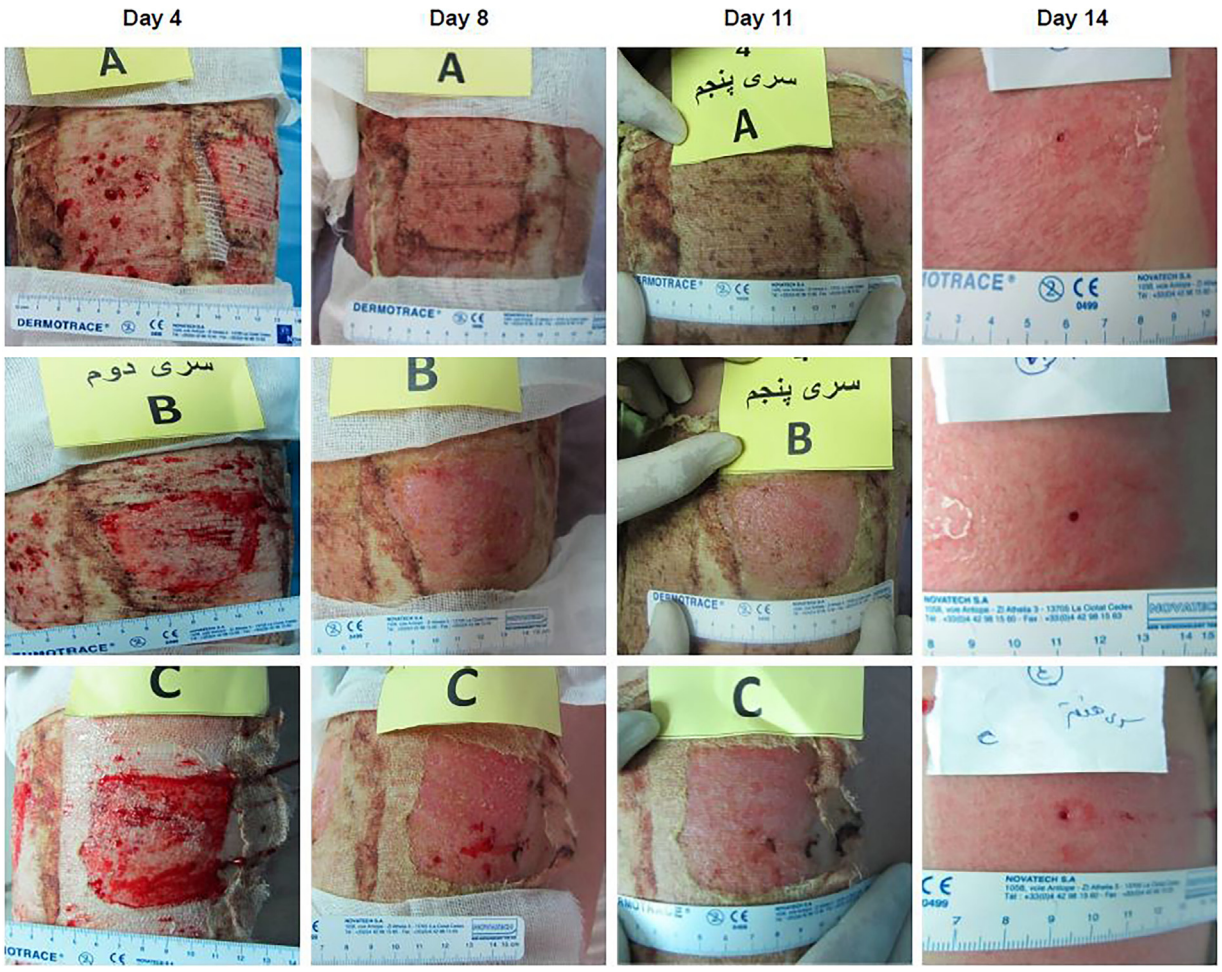

Fig. 4 – Macroscopic observation of re-epithelialization in patient number 4 during the days after treatment.
A: Control, B: amniotic membrane, C: amniotic membrane seeded with fetal fibroblasts.

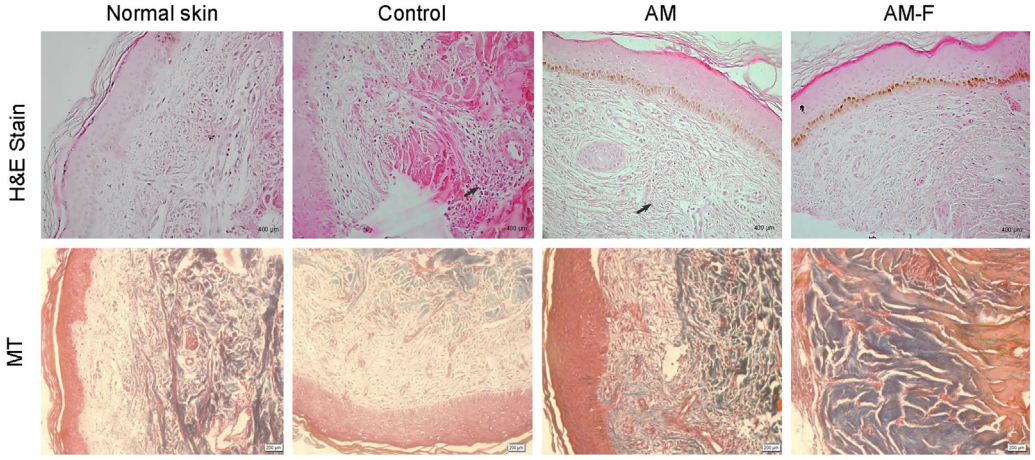

Fig. 5 – Histological findings of patient number 4. Punch biopsies obtained from normal skin and treatment sites were stained by H&E and Masson's trichrome (MT). Findings revealed accelerated wound regeneration and decreased inflammatory cells infiltration (indicated by arrow) in AM-F and AM groups compared to control group. Masson's trichrome staining displayed similar collagen staining in the treatment and control sites.
AM: amniotic membrane, AM-F: amniotic membrane seeded with fetal fibroblasts.

This could be explained by the following issues. In this phase I clinical trial, a limited number of patients were enrolled. Investigating the probable efficacy of amniotic membrane seeded with fetal fibroblasts on donor-site healing in burn patients may require larger randomized clinical trials. A previous study showed that whole skin substitutes (that comprise dermal and epidermal cells) could accelerate healing time and lessen scar formation in full-thickness wounds [20]. Also, a recent randomized, controlled, multi-center study showed a higher efficacy for bioengineered cell-based skin substitutes compared to acellular dermal templates on chronic diabetic wounds healing [21]. However, some studies suggested that cell-based whole-skin substitutes do not provide additional benefits over acellular templates in all cases [31,32]. Furthermore, donor sites are superficial wounds with least damage on dermal tissue. These wounds probably require epidermal cells rather than dermal cells for faster regeneration. In this regard, a recent study concerning the application of cultured autologous epidermis (CAE) on donor sites of 49 patients, showed rapid epithelialization within 7 days (ranging from 5 to 10 days) among treated sites and suggested epidermal transplantation in cases of donor sites' wounds [33]. Further studies should be conducted in order to compare the efficacy of acellular scaffolds with epidermal cell-based substitutes on donor site healing.

Our findings suggest amniotic membrane as a potential regenerative option as it accelerated donor area healing, lessened pain, and inflammation, and allowed more frequent graft harvest. Additionally, as amniotic membrane can be easily harvested and stored in any country, it is an economically reasonable alternative biomaterial for treatment of donor sites in developing countries. Although, this study showed that fetal cell-based skin substitutes were safe and they were well tolerated when applied to donor sites in burn patients, the beneficial effects of fetal fibroblasts on donor-site healing should be investigated in larger randomized clinical trials.

Conflict of interest

None of the authors has a financial interest in any of the products or drugs mentioned in this article.

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Author contribution

Mohammad Javad Fatemi, Nasser Aghdami, and Mahnoush Momeni conceived and designed the original protocol. All authors were involved in amending the protocol. Mahnoush Momeni, Nasrin Fallah, and Amir Bajouri conducted the study. Nasrin Fallah performed the cell and scaffold processing. Mohammad Javad Fatemi, Mahnoush Momeni, Amir Bajouri, Zahra orouji, Saeed Shafieyan, and Tooran Bagheri were involved in patient recruitment and follow-up visits. Mohammad Javad Fatemi and Mahnoush Momeni performed the surgery and substitute transplantation. Histological assessments were done by Niloofar Sodeifi. Data entry was performed by Tooran Bagheri and Parisa Pahlevanpour. Ahad Alizadeh cleaned and analyzed the data. Amir Bajouri wrote the first draft of the manuscript. Mohammad Javad Fatemi and Nasser Aghdami supervised the study. All authors read and approved the final manuscript.

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