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REGULAR ARTICLE

Healing potential of injectable *Aloe vera* hydrogel loaded by adipose-derived stem cell in skin tissue-engineering in a rat burn wound model

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Abstract

Adipose stem cells (ASCs) are a great promise in wound healing due to their potential in differentiating into various cell lineages and secreting growth factors. The purpose of this study is to evaluate the in vivo effects of *Aloe vera* hydrogel loaded by allogeneic ASCs on a rat burn wound model. The ASCs were isolated, cultured and mixed with 50% *Aloe vera* hydrogel and injected intradermally around the wound. Demineralized bone matrix (DBM) was used as dressing in the experiment. The burn wound-healing properties of different experimental groups were investigated by histopathological, molecular, scanning electron microscopic and biochemical analysis at the 7th, 14th and 28th days post-wounding. The *Aloe vera* and DBM-*Aloe vera* groups showed almost similar healing properties, while treatment by DBM-*Aloe vera*/ASCs significantly enhanced wound healing. The levels of transforming growth factor-β1 (TGF-β1) and interleukin-1β markedly decreased at the 7th day post-injury, in the DBM-*Aloe vera*/ASC-treated group, suggesting that this treatment regime subsided the inflammatory responses. Angiogenesis, re-epithelialization and the level of TGF-β1 in the wounds treated with DBM-*Aloe vera*/ASCs were also remarkably higher than those of other groups, at the 14th day post-injury. Besides, scar formation significantly decreased in the DBM-*Aloe vera*/ASC-treated wounds when compared with other groups. Our biochemical results were in agreement with the molecular and histopathological findings and strongly demonstrated that a DBM-*Aloe vera*/ASC composite can stimulate burn wound healing. These results suggest that the DBM-*Aloe vera*/ASC composite can be considered as a promising therapeutic strategy in the treatment of burn wounds.

Keywords Adipose stem cell · *Aloe vera* · Demineralized bone matrix · Burn wound model · Histopathology · Real-time PCR

Introduction

Wound healing is a dynamic and complex process that can be divided into three or four predictable phases including hemostasis, inflammation, proliferation and remodeling (Demidova-Rice et al. 2012). Many factors such as bacteria, oxygen tension and bleeding can compromise the healing process by prolonging the inflammatory phase, reducing growth factors and impairing neovascularization (Falanga 2005). In the meantime, burns are the most common form of skin injuries. Wound infection is one of the most important reasons of mortality in burn wounds (Oryan et al. 2017). In spite of recent advancements in the management of burn wounds, there is no appropriate and reliable method to heal burn wounds. Application of growth factors has been indicated as a modest satisfactory practice in promoting wound healing. However, high cost and difficult delivery methodologies are limitations to use growth factors in wound healing (Nie et al. 2011). To date, mesenchymal stem cells (MSCs) have mainly been studied in treating wounds in animal models (Lin et al. 2013; de Mayo et al. 2017; Di et al. 2017; Kato et al. 2017). The MSCs have been shown to be effective in enhancing wound healing

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by modulating the immune response, secreting paracrine factors and promoting angiogenesis, thereby providing the building blocks for wound regeneration (Gimble et al. 2007; Chen et al. 2008; Lawall et al. 2010).

Several studies have reported the effective role of bone marrow mesenchymal stem cells (BMSCs) in promoting wound healing (Fu et al. 2006). In a comparative study, Basiouny et al. (2013) showed that topical application of BMSCs is more effective than the injection method (Basiouny et al. 2013). Despite the advantages of BMSCs in wound healing, there are several limitations related to this technique. Topical application of BMSCs requires an adequate number of cells and a low yield of these cells from the bone marrow is one of the limitations of this method. Moreover, it has been demonstrated that by age advancement of the donor the differentiation potential of BMSCs significantly decreases (Rao and Mattson 2001). Hence, the adipose-derived stem cells (ASCs) have been considered for cell transplantation therapy in regenerative medicine.

The ASCs have several advantages including promoting angiogenesis, secreting growth factors and differentiating into multipotent cell types. Such criteria have made ASCs an attractive treatment modality in soft tissue regeneration (Cherubino et al. 2011). Due to these advantages, many studies have been undertaken to increase the proliferation and differentiation potential of ASCs. For this purpose, several synthetic and semi-synthetic substances including growth factors and recombinant cytokines have been used as proliferating and differentiating agents (Udalamaththa et al. 2016). These materials are excessively expensive and may also lead to side effects and toxicity. Hence, finding alternative natural products that in combination with ASCs result in enhanced proliferation and differentiation of ASCs is a priority. Previous works have shown promising effectiveness of herbal extract in differentiation and proliferation of human mesenchymal stromal cells (hMSCs) (Potu et al. 2009; Warrier et al. 2013; Aziz et al. 2015). Low toxicity and high availability are important reasons in using herbal remedies in proliferation and differentiation of MSCs. For instance, olive leaf extract can differentiate the hMSCs into endothelial cells, which is vital in angiogenesis and vasculogenesis processes (Gomez et al. 2012). Sholehvar et al. showed that *Aloe vera* gel significantly increased the viability of dental pulp stem cells (Sholehvar et al. 2016).

This study was designed to investigate the effects of ASCs combined with *Aloe vera* on burn wound healing in a rat model. We isolated the ASCs from subcutaneous adipose tissues of rat, combined the cells with *Aloe vera* and then applied the mixture on burn wounds (Fig. 1). In this experiment, we evaluated structural development, wound closure, re-epithelialization and wound contraction potential of the ASCs combined with *Aloe vera*, by gross morphologic, histopathologic and molecular investigations; scanning electron microscopy (SEM); and biochemical analysis during the inflammatory, fibroplasia and maturation phases of burn wound healing.

Materials and methods

Animals

Twelve 7–9-week-old Sprague-Dawley male rats weighing 200 ± 20 g were housed in separate cages under standardized diet, water, temperature, humidity and light (12:12-h light–dark cycle) conditions. After 1 week of an acclimatization period, the rats were used for the experiments. All the surgical procedures and experiments were performed according to the Guide for Care and Use of Laboratory Animals approved by the Institutional Animal Care and Use Committee of our veterinary school (approval number 14-6-1394 and approval date: 5 September 2015).

Isolation and culture of the rat (ASCs)

Adipose stem cells were isolated as previously described by Hsu and Hsieh (2015). Briefly, the adipose tissues were harvested from the subcutaneous tissue of allogenic rats and enzymatically digested with 0.1% type I collagenase (Sigma) at 37 °C for 1 h. After centrifugation at 700×*g* for 5 min, the cells were collected and plated in flask T-25 with Dulbecco's modified eagle medium-low glucose (DMEM)/F12 (1:1) (Gibco) containing 10% fetal bovine serum (Gibco) and 10 mg/L penicillin-streptomycin at 37 °C in a 5% CO_2 incubator. In order to remove the debris, the cells were washed with phosphate-buffered saline (PBS) after 24 h and fresh medium was then added. The ACSs were expanded in culture and passages 3–5 were used throughout the study.

Preparation of the *Aloe vera* hydrogel

After washing and disinfecting the *Aloe vera* leaf, the inner gel was separated and hemogenized. To prepare 50% (*v*/*v*) concentration, the inner gel was diluted by DMEM and then filtered, using a 0.45-μm filter mesh. In this study, we used 50% *Aloe vera* for our experiments in accordance with the results of Sholehvar et al. (2016) who showed that the ASCs in 50% *Aloe vera* hydrogel have higher viability (97.73%) than other concentrations (Sholehvar et al. 2016).

Preparation of demineralized bone matrix

To prepare demineralized bone matrix (DBM), the femur bone of a healthy 2-year-old Holstein cow was cut into pieces of 1–2-mm dimensions. The samples were placed in 0.5 M

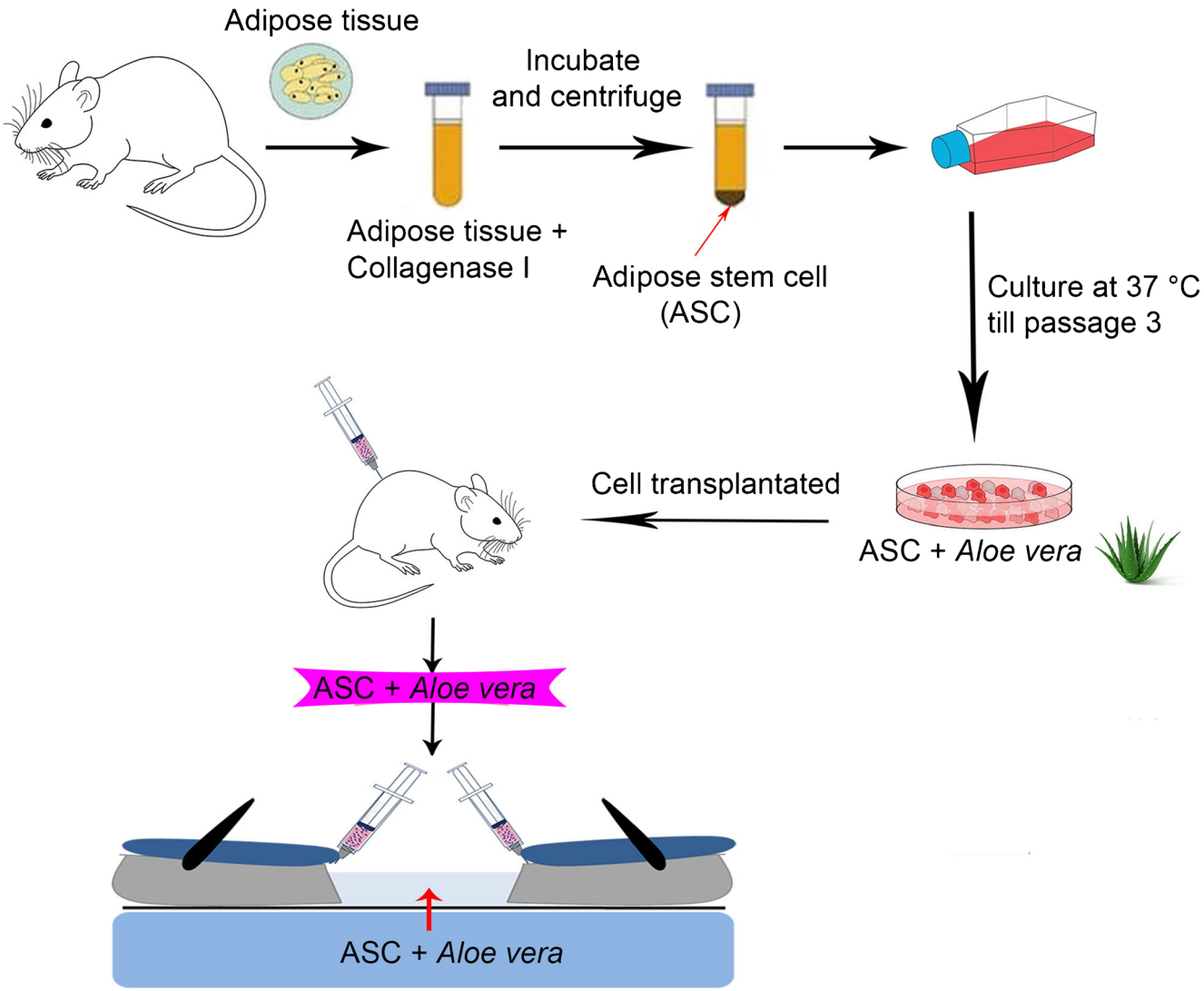

Fig. 1 Schematic diagram of the experimental procedure for transplanting the ASCs combined with *Aloe vera* in a burn wound-healing model in rat. ASCs adipose stem cells, DBM demineralized bone matrix

hydrochloric acid (Merck) at 4 °C under daily examination to be appropriately demineralized. Radiology was conducted to confirm sufficient demineralization in the samples. The samples were demineralized after 3 weeks. To sterilize, the samples were immersed in absolute ethanol for 24 h and were then rinsed with deionized distilled water and maintained at 4 °C (Bigham-Sadegh and Oryan 2015).

Cell viability assays

To evaluate the role of *Aloe vera* in protecting cells, a MTT assay was performed, according to the manufacturer's instructions (BIO-IDEA). Human dermal fibroblast (HDF) cells were cultured in 96-well culture plates at a density of 5×10^3 cells. After 24 h, the medium was removed and replaced by media containing 1% *Aloe vera* and incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 12 and 24 h. Ten microliters of the 12 mM MTT stock solution was then added into each well and incubated at 37 °C for 4 h. Afterward, 50 μL of DMSO was added to each well and thoroughly mixed by a pipette. The absorbance was read at 570 nm, using an ELISA reader, after 10-min incubation.

Burn wound model

The rats were anesthetized with an intramuscular injection of ketamine (10%, 75 mg/kg BW) and xylazine (2%, 10 mg/kg BW) (both from Alfasan Co., Woerden, Holland) and surgery was carried out under sterile condition. Four circular burn wounds 10 mm in diameter were created on the back of each rat by an aluminum bar. The aluminum bar was boiled in 100 °C water for 30 s and placed immediately on each area for 10 s without pressuring. After 48 h, the burned area was punched with a punch biopsy (Sun et al. 2011; Bhatia et al.

2016). Finally, each rat had four 10-mm diameter full thickness burn wounds on the dorsum. The debrided burn wounds were topically treated with one of the following treatment strategies:

1) DBM: The lesions received no treatment and were covered by DBM dressing.
2) *Aloe vera*: The lesions received 200 μL of 50% *Aloe vera* topically and 800 μL intradermally.
3) DBM-*Aloe vera*: The lesions received 200 μL of 50% *Aloe vera* topically and 800 μL intradermally and were covered by DBM dressing.
4) DBM-*Aloe vera*/ASCs: A total of 1×10^6 cells in 100 μL of 50% *Aloe vera* gel were used in each lesion, so that 0.7×10^6 cells in 60 μL of 50% *Aloe vera* were injected intradermally around the wound at four injection sites and 0.3×10^6 cells in 40 μl of 50% *Aloe vera* were applied onto the wound bed.

The animals were housed individually for 7, 14 and 28 days. Sampling was carried out after euthanizing the animals at 7, 14 and 28 days after wounding ($n = 4$/treatment/time point).

Rate of wound closure

To estimate the rate of wound closure, the lesions were photographed at 0, 7, 14 and 28 days post-wounding. The images were analyzed in Digimizer 4.2.6.0 to calculate the percentage of wound closure, using the following equation:

Percentage of wound closure = [(wound area on day 0 − wound area on indicated day) / wound area on day 0] × 100.

RNA isolation and quantitative real-time polymerase chain reaction analysis

After 7, 14 and 28 days of surgery, the samples were harvested to extract RNA for real-time polymerase chain reaction (RT-PCR) analysis. The RNA was isolated using a DenaZist commercial kit according to the manufacturer's instructions. In order to eliminate genomic DNA contamination, the RNA was treated with DNase I. Purity and concentration of the RNA was measured with gel electrophoresis and NanoDrop. The cDNA library was generated using the PrimeScript™ RT reagent kit (TaKaRa, Japan) following the manufacturer's instructions. RT-PCR was used to amplify interleukin-1β (IL-1ß), transforming growth factor-β1 (TGF-ß1), basic fibroblast growth factor (bFGF) and ß-actin, using SYBR Premix Ex Taq II (TaKaRa, Japan). The reaction was carried out in a Exicycler™ 96 Quantitative Real-Time PCR System (Bioneer) for 40 cycles. The following specific primers were used: IL-1ß forward: TCTGAAGCAGCTATGGCAAC and IL-1ß reverse: TCAGCCTCAAAGAACAGGTCA, TGF-ß1 forward: ACTACGCCAAAGAAGTCACC and TGF-ß1 reverse: CACTGCTTCCCGAATGTCT, ß-actin forward: TCCGTAAAGACCTCTATGCC and ß-actin reverse: GATAGAGCCACCAATCCACA, and bFGF forward: ATTTCCAAAACCTGACCCGAT and bFGF reverse: TGCCTTTTAACACAACGACCAG. The relative mRNA level of each gene of interest was normalized to ß-actin and calculated using the $2^{-\Delta\Delta CT}$ method (Tabandeh et al. 2014).

Histopathology

For histological analysis, the wounded area, including the epidermis, dermis and subcutaneous parts along with a thin portion from the surrounding intact tissues, were harvested at 7, 14 and 28 days post-wounding. The samples were fixed in 10% neutral buffered formalin, dehydrated by graded ethanol, cleared by xylol, embedded in paraffin and sectioned at 5 μm in thickness. Hematoxylin and eosin (H&E) staining was performed and the biopsies were analyzed for the evaluation healing process. The re-epithelialization rate was assessed by ImageJ and Photoshop software (ImageJ, NIH, CA, USA and Adobe Co., Photoshop CS 5 extended, NY, USA) on day 14 semiquantitatively. The epitheliogenesis scoring system is shown in Table 1. To evaluate the degree of inflammation, the number of polymorphonuclear and mononuclear inflammatory cells including neutrophils, lymphocytes, plasma cells and macrophages was counted. To evaluate angiogenesis and rate of granulation tissue, the new blood vessels and fibroblasts + fibrocytes were counted, respectively. Fibroblast proliferation, neovascularization and polymorphonuclear and mononuclear inflammatory cell infiltration were blindly assessed in three fields in each tissue section and the average number of each criterion was recorded (×400) (Oryan and Zaker 1998; Oryan et al. 2007, 2008, 2011, 2012).

Ultrastructural evaluation

To prepare the scanning electron microscopic (SEM) samples, the skin specimens were harvested on the 28th post-wounding day and fixed in cold 2.5% glutaraldehyde plus 2% paraformaldehyde with 2–5 mM calcium chloride in 0.1 M cacodylate buffer and then dehydrated in graded ethanol solutions. The samples were then freeze-dried at − 80 °C for 24 h and contrast was improved by coating the specimens with gold in a sputtering coating unit (Q150R- ES). Under SEM (TESCAN-Vega 3) high-qualified images were created and

Table 1 Epitheliogenesis scoring system at 14 days post-wounding

Without new epithelialization	0
25–50% epithelialization	1
50–100% epithelialization	2

the structure of collagen fibrils and fibers were assessed (Oryan et al. 2011).

Dry matter and hydroxyproline content

In order to calculate the percentage of dry matter, the samples were collected and immediately weighed at 7, 14 and 28 days post-surgery. The tissue samples were then freeze-dried and the percentage of dry matter was calculated by the following formula: percentage dry matter content = (dry weight / wet weight) × 100 (Oryan et al. 2009, 2015). The hydroxyproline content was also measured according to the manufacturer's instructions of the Kiazist commercial kit.

Data analysis and statistics

The quantitative data were statistically compared, using Kruskal-Wallis *H*, non-parametric ANOVA test and two-tailed Mann-Whitney *u* test. Statistics were performed using the computer software SPSS, version 19 (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was considered significant.

Results

In vitro evidence

The viability of HDF cells was determined using MTT assay, after 12 and 24 h (Fig. 2). One-percent *Aloe vera* was able to protect cells and increase the viability cells after 12 and 24 h compared to the control group.

Macroscopic changes in the wound site

The wounds were monitored at 7, 14 and 28 days post-wounding to evaluate redness, inflammation and hyperemia. The DBM-*Aloe vera*/ASC-treated wounds indicated no redness and inflammation at the 14th day post-wounding (Fig. 3d″). In contrast, the lesions in the DBM group showed severe inflammation and redness after 2 weeks of wound injury (Fig. 3a″). Dressings remained on the skin for 3 days and showed no significant difference between DBM-*Aloe vera*- and *Aloe vera*-treated groups. On the basis of our results, DBM had no any effective role in our study so that wounds treated with DBM-*Aloe vera* and *Aloe vera* showed the same results. The DBM-*Aloe vera* and *Aloe vera* groups also indicated moderate inflammation and redness at the 14th day post-wounding (Fig. 3b″, c″). There was no sign of redness, hyperemia and inflammation in the DBM-*Aloe vera*/ASC-treated wounds, after 28 days of surgery, while a mild redness and inflammation were still seen in the DBM, DBM-*Aloe vera* and *Aloe vera* groups at this time point (Fig. 3a‴–d‴).

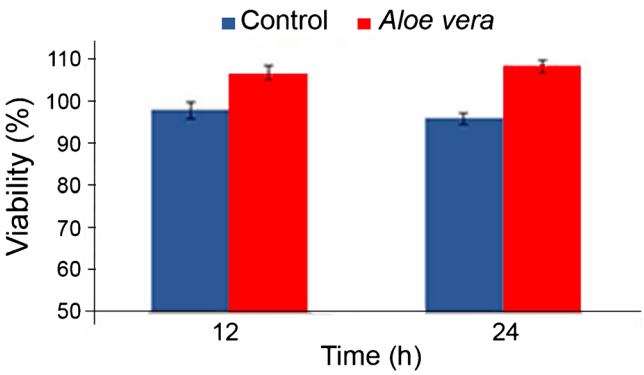

Fig. 2 Effects of *Aloe vera* on cell viability of HDF. HDF human dermal fibrobast

As seen in Table 2, the rate of wound closure was significantly higher in the DBM-*Aloe vera*/ASC group compared to the DBM, DBM-*Aloe vera* and *Aloe vera* groups at 7 and 14 days post-wounding ($p < 0.05$). The results showed no significant difference between the DBM-*Aloe vera* and *Aloe vera* groups at these time points. The DBM-*Aloe vera*/ASC group demonstrated 99.39 ± 0.6% wound closure at the 14th day post-wounding and was superior to other groups in new epidermis formation (Table 2 and Fig. 3d″; $p < 0.05$) at this stage. The *Aloe vera* and DBM-*Aloe vera* groups demonstrated 96.91 ± 0.8% and 96.89 ± 1.6%, wound closure rate, respectively and showed a significantly higher wound closure ratio in comparison to the DBM group ($p < 0.05$) at the 14th post-wounding day.

Gene expression level

To understand how the molecular mechanisms of *Aloe vera* containing ASCs improved burn wound healing, we compared the expression level of three key factors related to wound healing, including IL-1ß, TGF-ß1 and bFGF in lesions of the rats treated with DBM-*Aloe vera*/ASCs, DBM-*Aloe vera*, *Aloe vera* and DBM on the 7th, 14th and 28th post-operative days by RT-PCR. The DBM-*Aloe vera*/ASC-treated group expressed dramatically lower levels of IL-1ß and TGF-ß1 than other groups at day 7 post-injury. Furthermore, the levels of IL-1ß and TGF-ß1 were significantly lower in the groups containing *Aloe vera* than the DBM group ($p < 0.05$) (Fig. 4a, b). The expression level of IL-1ß gene was also significantly lower in the DBM-*Aloe vera*/ASC-treated wounds compared to the DBM, *Aloe vera* and DBM-*Aloe vera* groups, on the 14th post-injury day (Fig. 4a). There was no significant difference in the expression level of the IL-1ß gene between the DBM-*Aloe vera* and *Aloe vera* groups on the 7th and 14th post-surgery day ($p < 0.05$).

The wounds treated with DBM-*Aloe vera*/ASCs showed a significantly higher level of TGF- ß1 compared with other groups at 14 days post-wounding ($p < 0.05$; Fig. 4b). The TGF-ß1 expression then reduced at the 28th day in the DBM-*Aloe vera*/ASCs, DBM-*Aloe vera* and *Aloe vera* groups

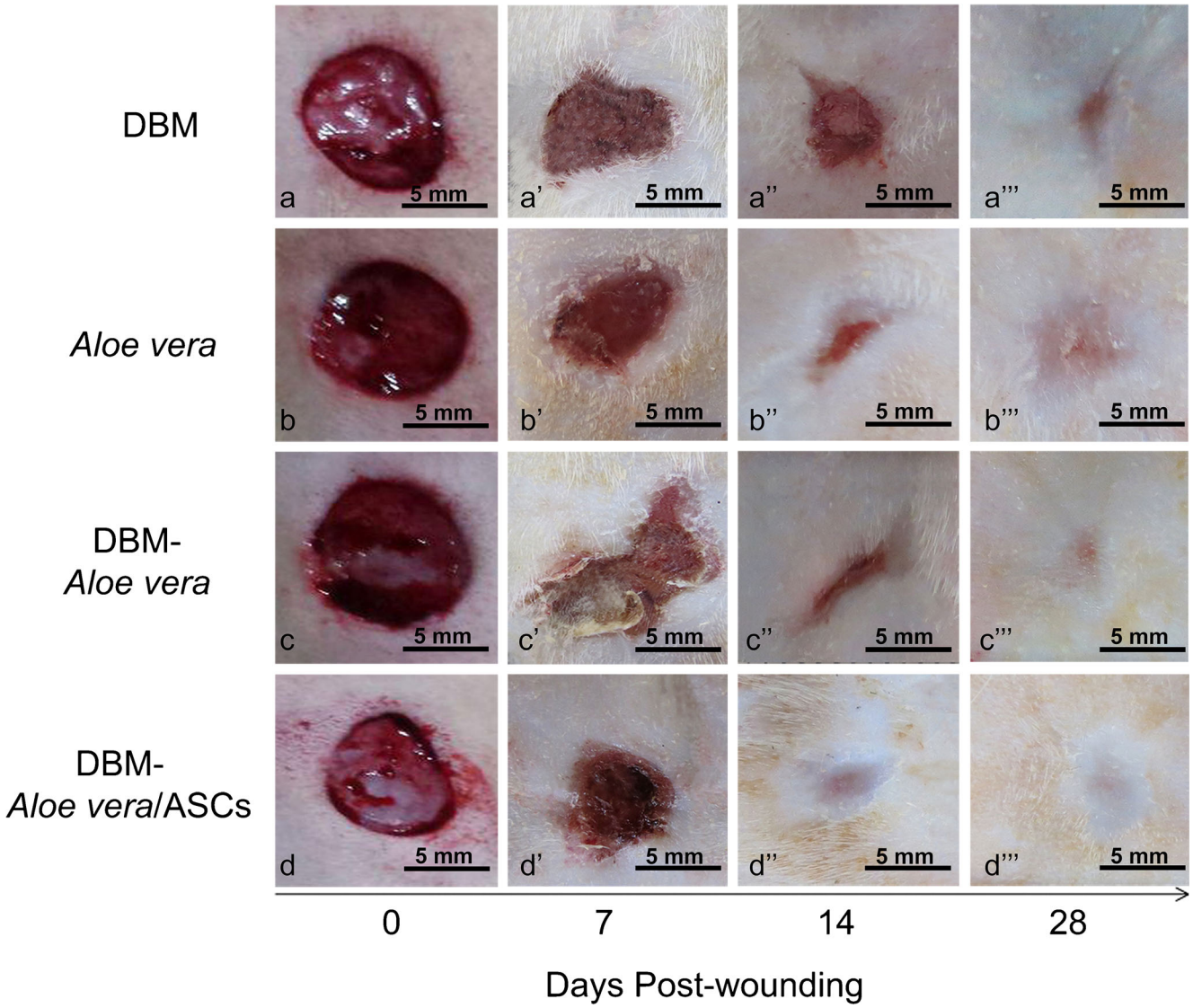

Fig. 3 Effects of DBM-*Aloe vera*/ASCs, *Aloe vera*, DBM-*Aloe vera* and DBM on the wound closure rate in burn wounds

when compared to the DBM group. The RT-PCR data in Fig. 4 indicate that treatment with ASCs significantly enhanced the expression level of bFGF at 14 and 28 days post-surgery ($p < 0.05$). The expression level of bFGF was also higher in

Table 2 Percentage of wound closure at 7, 14 and 28 days post-wounding (mean ± SD)

	7 day (Mean ± SD)	14 day (Mean ± SD)	28 day (Mean ± SD)
DBM	51.70 ± 5.43 *	81.77 ± 6.45 *	95.50 ± 0.84
Aloe vera	63.55 ± 0.21 *	96.91 ± 0.86 * *	99.35 ± 0.84
DBM-*Aloe vera*	56.90 ± 0.28 *	96.89 ± 1.67 * *	99.42 ± 0.84
DBM-*Aloe vera*/ASCs	69.05 ± 4.31	99.39 ± 0.61	100

DBM demineralized bone matrix, *ASCs* adipose stem cells

There was a significant increase ($p < 0.05$) in proportion of wound closure in the DBM-*Aloe vera*/ASC-treated group in comparison to other groups at the 7th and 14th post-wounding day

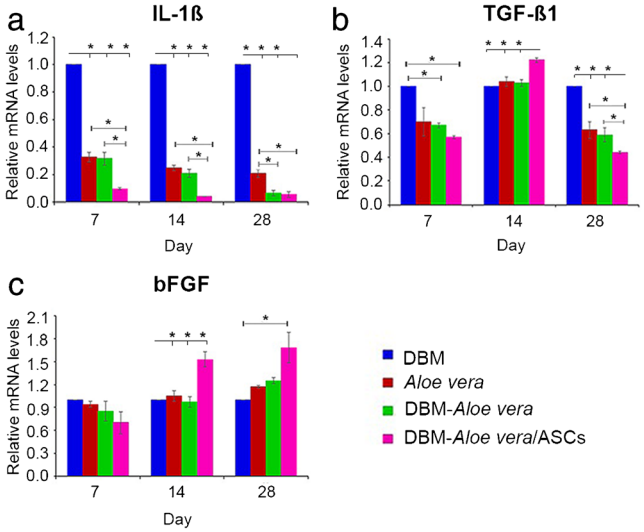

Fig. 4 Modulation of growth factors and cytokine profile by ASCs: mRNA level of **a** IL-1β, **b** TGF-β1 and **c** bFGF at various time points after burn injury, as determined by quantitative real-time RT-PCR

the DBM-*Aloe vera*/ASC-treated group in comparison to the DBM-*Aloe vera*, *Aloe vera* and DBM groups ($p = 0.02$, $p = 0.01$ and $p = 0.001$, respectively) at 14 and 28 days post-wounding. However, no significant difference was observed between the DBM-*Aloe vera*, *Aloe vera* and DBM groups at these time points. The RT-PCR results obtained here are in exceptionally good agreement with the histopathological and biochemical results.

Histological findings

Figure 5 illustrates the findings of the histological analysis of the wounds at the 7th, 14th and 28th days after wounding. Treatment by DBM-*Aloe vera*/ASCs, in particular, enhanced re-epithelialization compared with other treatment regimens at the 7th post-wounding day (Fig. 5a‴). There was no sign of epithelialization in the DBM-treated wounds at the 7th post-wounding day (Fig. 5a). The wound edges in the *Aloe vera*- and DBM-*Aloe vera*-treated groups were also covered by re-epithelialized epidermis at this time (Fig. 5a′, a″). Re-epithelialization was at its maximum in the DBM-*Aloe vera*/ASC-treated wounds and the injured area was completely covered by a tick epidermal layer compared to other groups at the 14th post-wounding day ($p < 0.05$; Fig. 5c‴ and Table 3). The DBM-, *Aloe vera*-, and DBM-*Aloe vera*-treated groups showed incomplete epithelialization at the 14th post-wounding day (Fig. 5c′, c″ and Table 3). The newly regenerated blood vessels were significantly more numerous in the DBM-*Aloe vera*/ASC-treated wounds in comparison to the DBM-, *Aloe vera*- and DBM-*Aloe vera*-treated wounds at 7 days after wounding ($p = 0.001$, 0.002, and 0.001, respectively) (Table 4). The DBM-, *Aloe vera*- and DBM-*Aloe vera*-treated wounds showed a similar trend in the number of microvessels at 7 days post-wounding (Table 4).

As seen in Table 4, DBM induced a more severe inflammatory reaction when compared to the other treatment strategies ($p < 0.05$) at 7, 14 and 28 days post-wounding. At these times, the wounds treated with DBM-*Aloe vera*/ASCs showed a significantly lower inflammatory cell count compared to those of the DBM-, *Aloe vera*- and DBM-*Aloe vera*-treated ones. There was no significant difference between the *Aloe vera* and DBM-*Aloe vera*-treated groups in term of inflammatory cell count at 7 days post-wounding (Table 4).

The wounds treated with the DBM-*Aloe vera*/ASCs showed a significantly higher number of fibroblasts and fibrocytes at the 14th post-wounding day, compared to the DBM-, *Aloe vera*- and DBM-*Aloe vera*-treated groups ($p = 0.001$, 0.002 and 0.003, respectively), while the difference between the DBM-*Aloe vera*- and *Aloe vera*-treated groups was not significant ($p = 0.67$) at this stage (Table 4). After 28 days of wounding, the number of fibroblasts and fibrocytes was significantly lower in the wounds treated with DBM-*Aloe vera*/ASCs than the DBM, DBM-*Aloe vera* and *Aloe vera* (Table 4).

The results obtained from histopathological examinations after 28 days of wounding disclosed more arranged and remodeled connective tissue bundles in the dermis of the DBM-*Aloe vera*/ASC-treated group compared with other groups (Fig. 5e‴, f‴). Overall, the DBM-*Aloe vera*/ASC-treated wounds demonstrated more cornification of the stratum corneum, more complete mature epidermis, higher fibroblasts + fibrocytes counts, lower inflammatory cell counts and a more organized arrangement of collagen fibers in the dermis, suggesting enhanced cutaneous regeneration.

Ultrastructural findings

Figure 6 illustrates the SEM findings at the 28th post-wounding day. The wounds treated by ASCs showed a better alignment of collagen fibrils compared to those of the other groups. Actually, the aligned collagen fiber bundles can be a reason for improved tissue remodeling and maturation.

Dry matter and hydroxyproline content

Hydroxyproline content as an indicator of collagen level plays an important role in accelerating the rate of wound closure. To determine whether ASCs are able to alter the content of collagen in the wound, we measured the dry matter and hydroxyproline level in the wounds. Table 5 indicates the hydroxyproline and dry matter content at the 7th, 14th and 28th days after injury. The hydroxyproline content and dry matter quantity were significantly higher in the wounds treated with DBM-*Aloe vera*/ASCs ($p < 0.05$) in comparison to other groups at day 14 post-wounding. These results are parallel to our histology and molecular findings in which exposure to ASCs

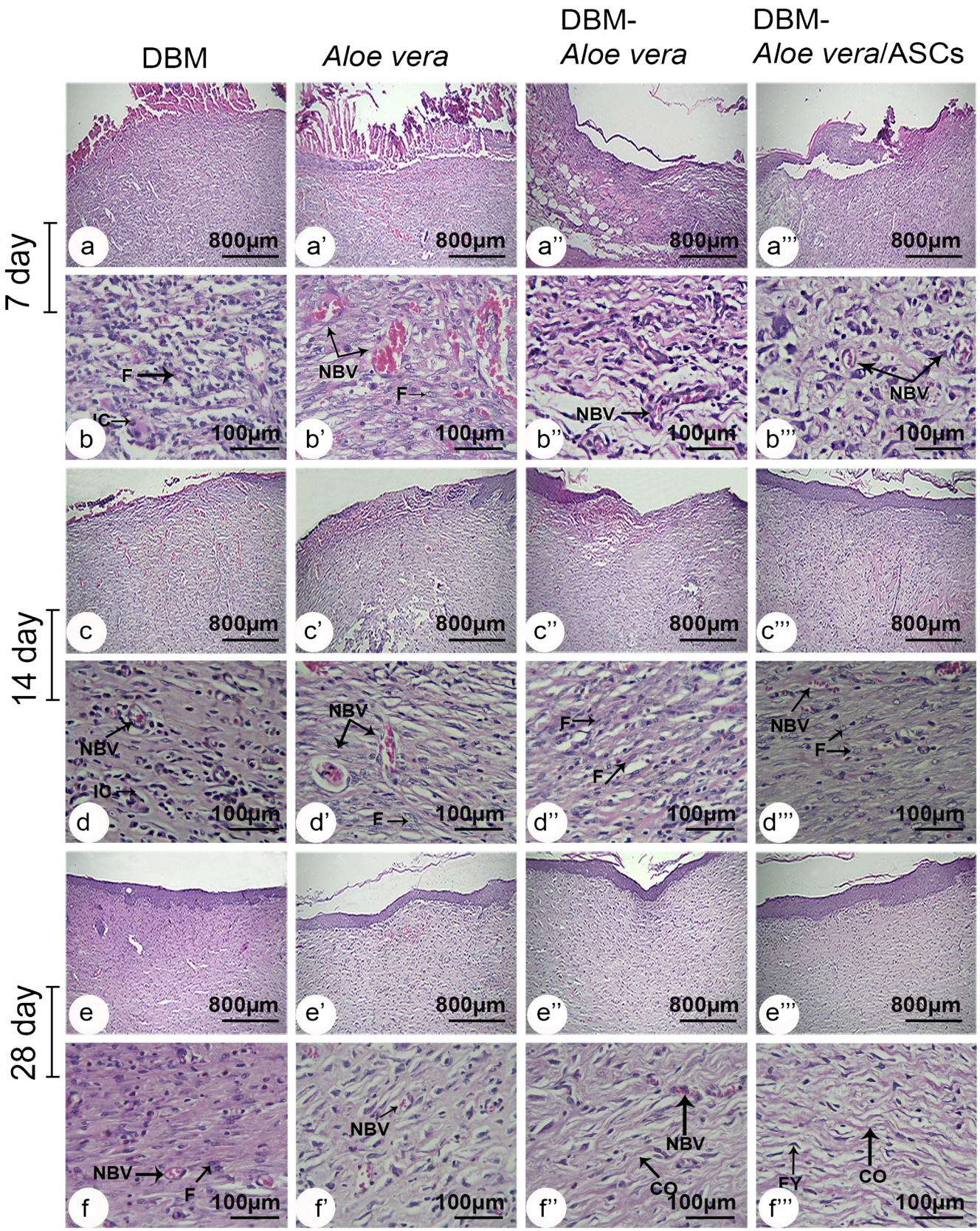

Fig. 5 Histology of the wounds treated with DBM, DBM-*Aloe vera*, *Aloe vera* and DBM-*Aloe vera*/ASCs on the 7th, 14th and 28th days after induction of burn wounds (hematoxylin and eosin, ×40 and ×400). IC inflammatory cell, NBV newly formed blood vessel, F fibroblast, CO collagen fiber, FY fibrocyte, HFC hair follicle

Table 3 Histomorphometric analysis of different experimental groups

Groups	Epitheliogenesis score ($n = 4$)
DBM	0,0,0,1
Aloe vera	1,0,1,0
DBM-*Aloe vera*	0,1,0,1
DBM-*Aloe vera*/ASCs	2,1,2,2*

*Values indicate DBM-*Aloe vera*/ASC group versus other groups, $p < 0.05$

stimulated wound contraction due to an increased collagen level at the 14th post-injury day.

Discussion

This study indicates that a treatment containing adipose stem cells is an optimal option in healing burn wounds. Combination of *Aloe vera* and ASCs resulted in a less inflammatory response in comparison to the groups without ASCs. *Aloe vera*/ASCs also promoted angiogenesis and granulation tissue formation through increased expression of bFGF and TGF-ß1 after 14 days of injury. The skin in the DBM-*Aloe vera*/ASC-treated group showed complete re-epithelialization and improved cosmetic appearance compared to other experimental groups at 14 days post-wounding. After 28 days of wounding, treatment containing ASCs resulted in higher expression of bFGF, which was responsible for inducing anti-scaring function. These findings were supported by demonstrating a more organized tissue architecture, more fibroblast maturation to fibrocytes at the histopathological level and presence of more hydroxyproline and dry matter content in the lesions of the ASC-treated animals.

In this study, we designed two groups comprising DBM-*Aloe vera* and *Aloe vera* to investigate the interfering effects of DBM on the healing process. Although DBM somewhat improved the healing process, our results showed no significant difference between DBM-*Aloe*- and *Aloe vera*-treated groups. On the basis of our results, DBM had no any significant effective role in our study so that wounds treated with DBM-*Aloe vera* and *Aloe vera* showed the same results. Comparing the DBM-*Aloe vera*- and DMB-*Aloe vera*/ASC-treated group, we observed a significant improvement over other groups, which is related to ASCs. Since the role of ASCs has been confirmed by many researchers, our main aim was to show the effective role of hydrogels in protecting ASCs in wound environment. Our results indicated that there are significant differences between DBM-*Aloe vera* and DMB-*Aloe vera*/ASCs even at 14 and 28 days post-wounding. On the basis of this result, we believed that *Aloe vera* could protect ASCs against oxidative stress. To confirm our theory, we evaluated the viability of human dermal fibroblast cells to protect in the presence of *Aloe vera*. MTT results showed that cells treated with *Aloe vera* could protect cells and increase the viability compared to the control group. This result confirmed our theory that *Aloe vera* hydrogel can protect ASCs against oxidative stress and increase the viability of cells. The positive effects of

Table 4 Histomorphometric findings in wounds treated with DBM, *Aloe vera*, DBM/*Aloe vera* and DBM-*Aloe vera*/ASCs after 7, 14 and 28 days of injury in rats

		Blood vessels (*n*)	Fibrocytes + fibroblasts (*n*)	Inflammatory cells (*n*)
7 days	DBM	5.30 ± 0.40	48.20 ± 6.40	43.45 ± 1.60
	Aloe vera	9.90 ± 0.40	61.37 ± 4.30	10.95 ± 0.90*
	DBM-*Aloe vera*	5.50 ± 0.70	43.95 ± 0.40	9.21 ± 0.90*
	DBM-*Aloe vera*/ASCs	17.20 ± 3.30*	35.10 ± 0.70	4.25 ± 1.00*
	DBM	6.80 ± 0.70	58.30 ± 4.60	23.40 ± 1.80
	Aloe vera	6.80 ± 1.10	65.80 ± 3.90	12.80 ± 1.10*
14 days	DBM-*Aloe vera*	4.60 ± 0.00	68.90 ± 0.80	5.76 ± 0.70*
	DBM-*Aloe vera*/ASCs	8.80 ± 0.70*	88.60 ± 2.30*	2.50 ± 0.70*
	DBM	2.80 ± 0.90	58.02 ± 2.90	5.10 ± 1.50
	Aloe vera	3.10 ± 0.60	34.30 ± 3.80*	2.15 ± 0.40*
28 days	DBM-*Aloe vera*	1.30 ± 1.10	25.60 ± 0.80*	2.84 ± 0.80*
	DBM-*Aloe vera*/ASCs	0.30 ± 0.00*	19.22 ± 1.90*	0.40 ± 0.10*

The number of inflammatory cells significantly decreased in *Aloe vera*, DBM-*Aloe vera* and DBM-*Aloe vera*/ASC groups compared with the DBM group ($p < 0.05$) after 7, 14 and 28 days post-surgery. There was no significant difference between *Aloe vera* and DBM-*Aloe vera*. At 7 days, the number of blood vessels significantly increased in the DBM-*Aloe vera*/ASC group compared with other groups ($p < 0.05$). There was a significant increase in the number of fibrocytes + fibroblasts in the DBM-*Aloe vera*/ASC group when compared to other groups ($p < 0.05$) after 14 days of operation. At 28 days, the number of fibrocytes + fibroblasts significantly decreased in the *Aloe vera*, DBM-*Aloe vera* and DBM-*Aloe vera*/ASC groups compared with the DBM group ($p < 0.05$)

*$p < 0.05$

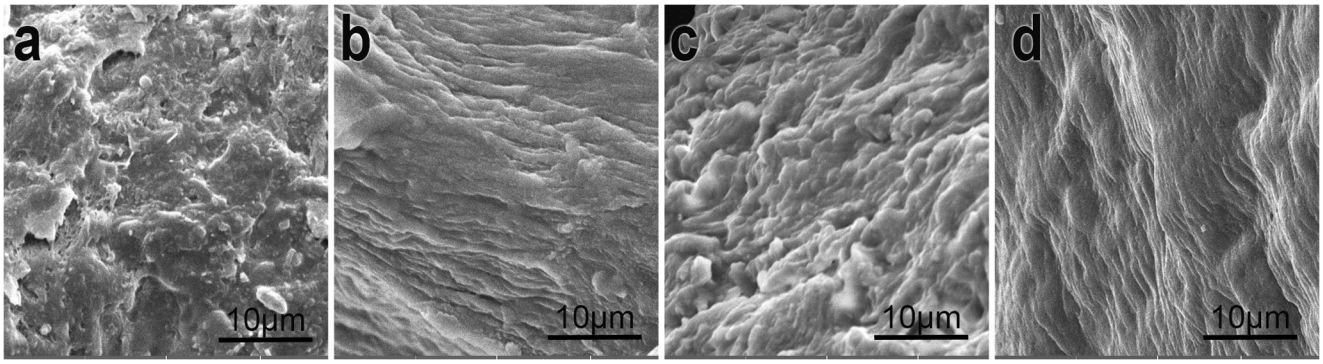

Fig. 6 Scanning ultrastructural findings after 28 days post-surgery. **a** DBM, **b** *Aloe vera*, **c** DBM-*Aloe vera* and **d** DBM-*Aloe vera*/ASCs. Treatment with DBM-*Aloe vera*/ASCs improved alignment of collagen fibrils compared to those of other groups. ASCs adipose stem cells, DBM demineralized bone matrix

ASCs at 14 and 28 days were a reason for protecting effect *Aloe vera* from cells during healing phases.

Nutrient constituents in *Aloe vera* such as acemannan, sugars, amino acids, vitamins, calcium, magnesium and potassium are responsible for the cytoprotective effects of *Aloe vera* gel. Proper physiological pH and osmolarity are other *Aloe vera* properties that maintain the viability of cells (Joseph and Raj 2010). Accordingly, *Aloe vera* is able to protect ASCs against free radicals and increase survival of these cells in the wound environment. It also seems possible that *Aloe vera* might act synergistically with ASCs because of the positive role of *Aloe vera* in stimulating growth factors and angiogenesis (Ferrara 2002; Pandya et al. 2006).In the present study, *Aloe vera*/ASCs enhanced capillary density and improved angiogenesis. A possible explanation for overproduction of blood vessels is due to differentiation of ASCs by *Aloe vera* into endothelial cells. It seems that angiogenesis at earlier stages of wound healing induced proliferation, migration and differentiation of ASCs and resulted in enhanced procollagen and tropocollagen synthesis and granulation tissue formation. It is obvious that the regenerating tissue commonly needs blood flow to supply oxygen and nutrients and remove wastes (Ko et al. 2007). In this regard, Jittapiromsak et al. (2010) showed that acemannan, an extracted product from *Aloe vera*, is able to stimulate dental pulp cell and endothelial cell proliferation and differentiation and promote dentin formation (Jittapiromsak et al. 2010). Ebrahimian et al. (2009) also demonstrated that ASCs are able to differentiate into kerationcytes and induce production of vascular endothelial growth factor and keratinocyte growth factor (Ebrahimian et al. 2009). Increased bFGF expression in the *Aloe vera*/ASC-treated group, at 14 days post-wounding, was another possible explanation in promoting angiogenesis.

Severe inflammatory cell infiltration in the wound area inhibits the repair and regeneration processes and induces more scar formation in wounds (Rosique et al. 2015). Many studies have reported the anti-inflammatory activity of ASCs and *Aloe vera* in various wound models (González et al. 2009; Oryan et al. 2010, 2016; Wang et al. 2016). The RT-PCR findings, in the present study, also suggested that treatment by *Aloe vera*/ASCs resulted in reduced inflammation by

Table 5 Effect of combination therapy of ASCs and *Aloe vera* on the hydroxyproline and percentage dry matter content in the wound environment on different days after wounding

Treatments	Dry matter (%)			Hydroxyproline (μg/mg dry wt. skin)		
	7th day, mean ± SD	14th day, mean ± SD	28th day, mean ± SD	7th day, mean ± SD	14th day, mean ± SD	28th day, mean ± SD
DBM	30.66 ± 5.18	32.33 ± 2.35	24.87 ± 8.51	19.30 ± 3.78	21.05 ± 0.78	28.47 ± 3.83
Aloe vera	30.41 ± 8.36	26.46 ± 1.60	31.83 ± 4.00	26.52 ± 0.86	22.82 ± 2.55	17.71 ± 1.93
DBM-*Aloe vera*	29.45 ± 1.11	21.50 ± 2.82	26.37 ± 2.29	23.12 ± 1.66	24.01 ± 1.17	21.98 ± 2.89
DBM-*Aloe vera*/ASCs	26.71 ± 1.81	37.50 ± 2.12*	25.66 ± 1.45	19.71 ± 0.28	28.42 ± 1.40*	14.67 ± 0.20*

Treatment by DBM-*Aloe vera*/ASCs significantly increased the hydroxyproline and percentage dry matter content when compared with the DBM group at 14 days post-wounding ($p < 0.05$). However, there was no significant difference between the *Aloe vera*- and DBM-*Aloe vera*-treated groups. In addition, there was a significant decrease in hydroxyproline content in the DBM-*Aloe vera*/ASC-treated group compared to the DBM group at 28 days post-wounding ($p = 0.02$)

DBM demineralized bone matrix, ASCs adipose stem cells

*$p < 0.05$

downregulating the IL-1β and TGF-β1 proinflammatory cytokine and growth factor. According to the Manning et al. (2015) findings, there is little evidence to support the hypothesis that ASCs can deactivate the production of IL-1β directly. They suggested that ASCs were able to modulate activity of macrophages and promote their switching into the M2 phenotype, which is a possible explanation in reducing the amount of pro-inflammatory cytokines and growth factors such as IL-1β and TGF-β1 (Manning et al. 2015).

The wounds treated by *Aloe vera*/ASCs, in the present in vivo study, demonstrated significant elevation in the number of fibroblasts at 14 days post-surgery. Fibroblasts are the main cells in the dermis that release various cytokines, growth factors, glycosaminoglycan, collagen type III and elastin that are known as the main constituents of repair and regeneration (Postlethwaite et al. 1987). Treatment by *Aloe vera*/ASCs, in the present study, promoted re-epithelialization and stimulated granulation tissue formation by enhancing the expression levels of bFGF and TGF-β1 at 14 days post-wounding. *Aloe vera* also had a positive role in stimulating the bFGF and TGF-β1 growth factors at this time, suggesting its effective role in wound regeneration. Hence, a combination of *Aloe vera* with ASCs might have acted synergistically and accelerated wound healing. Another possible explanation for improved repair in the *Aloe vera*/ASC-treated wounds is the effectiveness of *Aloe vera* in stimulating proliferation and differentiation of the ASCs and subsequently increasing the production of growth factors in the wound environment. These results were in agreement with our biochemical findings that showed a higher level of hydroxyproline in the *Aloe vera*/ASC-treated group on day 14 post-wounding.

Importantly, the expression of TGF-ß1 and hydroxyproline content diminished in the *Aloe vera*/ASC-treated group at 28 days post-wounding, which may be a possible explanation for anti-scarring activity and better organization of collagen fibers. Inhibition of excess hydroxyproline formation could be a reason for a significant decrease in the level of collagen in the ASC-treated wounds of this study. In agreement with our findings, Zhang et al. (2015) showed that ASCs decreased collagen type I, suggesting that ASC injections can suppress the formation of hypertrophic scars. In another study, Castiglione et al. (2013) showed that deposition of ECM components, such as collagen type I and III and elastin, decreased after treatment of scars with ASCs. In fact, ASCs, by reducing the level of hydroxyproline and collagen deposition, are able to decrease scars. This is consistent with results obtained in our molecular findings. ASCs can suppress fibrosis by various mechanisms, including reducing the expression of TGF-β1 and promoting the expression of bFGF, thus accelerating the turnover of the extracellular matrix. By increasing the bFGF level on day 28 post-wounding in the *Aloe vera*/ASC-treated wounds the anti-scarring role of the *Aloe vera*/ASC combination was clarified. Altogether, due to the effective role of ASCs on decreasing TGF-ß1 and increasing bFGF at 28 days post-wounding, this treatment modality could be a promising therapeutic strategy to be applied in clinical cases to prevent the exacerbated wound-healing processes and exaggerated scar tissue formation. Another likely function of ASCs in inhibiting scar formation is their role in reducing the inflammatory response in the wound environment and subsequent reduction of the pro-fibrotic responses that occur along with prolonged inflammation during wound healing (Redd et al. 2004; Jackson et al. 2012).

In this study, although we try to indicate the effective role of *Aloe vera* as a protecting agent, some limitations are worth noting. An important question for future studies is to compare the groups receiving ASCs with groups receiving ASCs/*Aloe vera*. Future investigations should therefore include follow-up work designed to evaluate the role of *Aloe vera* in protecting ASCs in detail and also compare wounds treated with *Aloe vera*-ASCs with ASCs alone during the wound-healing process.

Conclusion

This study demonstrated that a combination of ASCs and *Aloe vera* can effectively improve burn wound healing by stimulating mesenchymal cell proliferation, angiogenesis and re-epithelialization. In addition, *Aloe vera* amplified the anti-inflammatory effect of ASCs by reducing the TGF-ß1 and bFGF expression level and resulted in reduced scar formation. Combination of ASCs with *Aloe vera* hydrogel seems to be a step forward in the field of regenerative medicine since this strategy may have the potential to promote the proangiogenic effect of the ASCs and enhance burn wound healing. Combination of *Aloe vera* and ASCs formed a novel hydrogel scaffold in which incorporation of *Aloe vera* with ASCs significantly enhanced the expression level of cytokines and growth factors and finally resulted in improved wound repair and regeneration.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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