IJMCM
Summer 2020, Vol 9, No 3
DOI: 10.22088/IJMCM.BUMS.9.3.234

Original Article

Differential Biological Behavior of Fibroblasts and Endothelial Cells under *Aloe vera* Gel Culturing

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Submmited 17 August 2020; Accepted 6 October 2020; Published 10 November 2020

Aloe vera is used for its large variety of biological activities such as wound healing, anti-fungal, anti-inflammatory, hypoglycemic, immunomodulatory, gastroprotective, and anti-cancer. Although the beneficial effects of *Aloe vera* on wound healing have been proven, little is known about its effects at the cellular level. In this study, we evaluated the angiogenic and migrative effects of *Aloe vera* gel on fibroblasts and endothelial cells. Fibroblasts and endothelial cells were cultured in monolayer conditions with low glucose DMEM with 10% serum and 1% penicillin-streptomycin. Fresh and mature leaves of *Aloe vera* were used for gel preparation. Cell proliferation and morphology were studied by an inverted microscope. The migration of fibroblasts was assessed by scratch assay. MTT assay was performed for cell viability assessment, and real-time RT-PCR was used for evaluation of *PECAM-1*, integrin α1 and β1 transcription. After two days, the protein level of PECAM-1 was detected by flow cytometry. Our results showed that *Aloe vera* has a higher proliferative effect on fibroblasts in comparison with endothelial cells. *Aloe vera* also induced the migration of fibroblasts. The viability of both types of cells was similar to control ones. Integrin α_1, β_1 and *PECAM-1* gene expression increased significantly ($P < 0.005$) in *Aloe vera* treated fibroblasts and endothelial cells in comparison with the control groups. However, the expression of these genes was significantly higher in fibroblasts in comparison with endothelial cells. Protein levels of PECAM-1 showed no change in both cell types upon *Aloe vera* treatment. *Aloe vera* gel induced angiogenic and cell adhesion properties in fibroblasts more than endothelial cells. Further investigations are needed to show the main role of fibroblasts rather than endothelial cells in wound healing by *Aloe vera* administration.

Key words: *Aloe vera* gel, fibroblast, endothelial cells, integrin, *PECAM-1*

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[DOI: 10.22088/IJMCM.BUMS.9.3.234]

Wound healing is a dynamic process that is directed by multiple cell types in four phases, hemostasis, inflammation, proliferation, and remodeling(1). Resident cells of the tissue as well as recruited cells participate in the healing process, and these local epithelial cells, fibroblasts, dendritic cells, and endothelial cells secret angiogenic factors (2). Fibroblasts also produce extra cellular matrix (ECM) for promoting angiogenesis (3). Each healing cell type and angiogenic factor have unique function time points for the healing process; for example, stimulation and migration of endothelial cells occur in the proliferation phase (2). Angiogenesis occurs in the proliferation phase to compensate for vascular injury and accelerate the healing of wounds (4). It has also been found that cluster of differentiation 31 *(CD31)*also known as Platelet endothelial cell adhesion molecule 1 (*PECAM-1*) is involved in endothelial cell migration and angiogenesis (5).

Integrins play an important role in wound healing, and help to express the genes associated with this process (6). Cell surface integrins are also essential for cell migration (7), proliferation, and angiogenesis (6). Integrins α1β1 are expressed in microvascular endothelial cells (8), and they provide the essential support for angiogenesis and migration of these cells (9). Antibody against integrin α1 inhibited fibroblast migration in collagen substratum (10). Integrins α1β1 and α2β1 are known as a major receptor in regulating ECM remodeling (11). Matrix remodeling is vital for wound healing. It has been found that integrins play a crucial role in re-epithelialization due to increasing proliferation in keratinocytes. Integrins control cell migration via the ECM as well (12).

Medicinal plants are used for accelerating wound healing, and *Aloe vera* (*Aloe barbadensis Miller* from Liliaceae family) is one of the most useful plants (13). Traditionally, it is used for a large variety of biological activities such as wound healing, anti-fungal activity, anti-inflammatory activity, hypoglycemic, immunomodulatory, gastroprotective, anti-cancer (14), and antibacterial effects (15, 16). It is believed that these activities of *Aloe vera* are due to the synergistic action of many bioactive compounds present in the gel (17). The gel is the inner layer of *Aloe vera* leaves, which contains amino acids, anthraquinones, carbohydrates, chromones, dietary fibers, enzymes, hormones, minerals, sterols, proteins, and organic compounds (18).

Anthraquinones are derivatives of anthracene from the quinone group, and are mainly recognized as laxative compounds. Recently, studies have shown that anthraquinones have antioxidant, antiviral, cytotoxic, and anti-inflammatory effects on squamous cell carcinoma cells and a bacteriostatic effect on *Streptococcus*. Aloin and aloe-emodin are the two main anthraquinones of *Aloe vera* (14). Aloin accelerates wound healing by increasing the proliferation of endothelial cells and fibroblasts and also inducing the expression of epidermal growth factor (19).

Carbohydrates include monosaccharides and polysaccharides. Acemannan is one of the most important polysaccharide compounds found in leaf gels of *Aloe vera*. These compounds have immunomodulatory, anti-cancer, antioxidant, wound healing, and neuroprotective effects (20). Another polysaccharide of *Aloe vera* is a water-soluble compound called glucomannan, which can increase fibroblasts proliferation, and accelerate wound healing (21). Today, glucomannan is used as a supplement to lose weight, regulate cholesterol levels, and treat constipation, diabetes, and atherosclerosis. New research showed that glucomannan of *Aloe vera* can also be used as an anti-tumor compound (22). Enzymes include catalase, amylase, oxidase, cellulase, lipase, and carboxypeptidase. Calcium, chlorine, chromium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc are the main mineral compounds of *Aloe vera* gel (22). *Aloe vera* has 4

[DOI: 10.22088/IJMCM.BUMS.9.3.234]

sterols, lupeol, sitosterol, cholestrol, and campesterol. These sterols have anti-inflammatory properties. Lupeol also has antiseptic and analgesic effects (23).

Acceleration of wound healing has occurred by oral and local administration of *Aloe vera* gel (24). It had shown that the oral administration of *Aloe vera* gel increased angiogenesis and accelerated the acute radiation-delayed wound healing process (25). In wound healing, *Aloe vera* promotes epidermal keratinocytes proliferation and migration (26). In corneal inflammation, transplantation of human corneal endothelial cells plays an important role in the regeneration of the cornea (27). Although these cells have limited capacity to proliferate (28), *Aloe vera* decreases inflammation by the release of IL-1β from human corneal cell line (27). Aloe-emodin, one of *Aloe vera* constituents was shown to increase white blood cells count after 6 weeks in fish (29). *In vivo* studies showed that wound healing was promoted using the mesenchymal stem cell (MSCs) (30). When adipose-derived stem cells were loaded on *Aloe vera* gel, burn wounds healed with decreased scar formation (31). We previously found that *Aloe vera* /collagen increased *PECAM-1* and integrins $\alpha_1\beta_1$ in adipose stem cells (32). A vast majority of studies illustrated the therapeutic activity of *Aloe vera* on the regeneration of tissues (16). Since little is known about the effect of *Aloe vera* on the different cells of a tissue, this study aimed to evaluate the proliferative, angiogenic, and migrative effect of *Aloe vera* gel on fibroblasts and endothelial cells, and to compare the gene expression level of *PECAM-1*, integrin α1β1in these cell lines.

Materials and methods

Aloe vera gel preparation

Aloe vera gel was extracted from a fully mature *Aloe vera* plant as a previously published method (33). In brief, the mature leaves were removed after surface washing, and their shell was removed under clean conditions. White pulp *Aloe vera* gel was homogenized by a mixer and centrifuged at 12,000 rpm for 30 min at 4 °C to divide the fibers. The supernatant was transferred into a new falcon, and sterilized with chloroform (10% gel volume) and stored in a refrigerator.

Cell culture

Human fibroblast cell line IBRC C10003 and endothelial IBRC C10638 were purchased from Pasteur Institute Cell Bank, Iran. Fibroblasts and endothelial cells were cultured at 37 °C, 5% CO2 in low glucose DMEM (Gibco, UK) with 10% placental serum. After 24 h, the culture media was replaced, and then the culture medium was changed every 2–3 days. During the cell culture, the morphology of cells was determined microscopically. The cells were trypsinized (0.25% trypsin/0.2% EDTA; Sigma, USA) and counted. Every 70-100 thousand cells were transferred to a flask, and 5 ml medium was added to them (32).

Hematoxylin and eosin staining

The cells (4000 cells/cm^2) were cultured on the coverslip glass, in 6 well plates. In the first step, the supernatant has been removed from the cells, and the cells have been washed by PBS and fixed with 4% paraformaldehyde after emptying the PBS, and washed once with PBS for one min.

To perform the hematoxylin and eosin (H&E) staining, cells on coverslips have been transferred to hematoxylin solution for 10 min, and after washing were immersed in eosin solution for 15 s. At the end, coverslips were inversely mounted on slides, and studied under the microscope. The cells were stained three times (34).

Cell viability evaluation

The cells of all groups were cultured in 96-well plates (3000 cells per well), and a working medium containing *Aloe vera* (10%) was added to the treated cells group. After 24 h, culture media was removed and 200 µl serum-free DMEM medium and 20 µl 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution

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(5 mg/ml, Sigma USA) were added to the cells. After 4 h, 100 µL dimethyl sulfoxide (DMSO, Sigma, Germany) was added to the chambers to dissolve the formazan crystals. After 30 min in a dark room, the optical density was recorded by ELISA reader at 540 nm (32). This experiment was repeated three times.

Scratch test

Fibroblasts were cultured by the mentioned method until 90% confluency was reached. At this point, the fibroblasts were split into 2 groups and *Aloe vera* gel was added to the experiment group. A part of the pellet was scratched using a sampler head. Cells were incubated for 24 h, and then the number of migrated fibroblasts was determined using a microscope. This experiment was conducted three times (35).

RNA extraction, cDNA generation, and quantitative reverse transcriptase-polymerase chain reaction (real-time RT-PCR)

The RNX-Plus solution (phenol + guanidine isothiocyanate) for total RNA extraction was the product of SinaClon (Cat.no: PS4131). In brief, 24 h after treatment, the culture medium was removed from groups, and total RNA was extracted from cells using the RNX-Plus Solution Kit, according to the manfacturer's protocol. After purification and quantification, RNA concentration was determined by measuring the optical density at 260 and 280 nm using Nanodrop (Nanodrop- ND-1000, Thermo Fisher Scientific, USA). PrimeScript reagent kit (Cat.no: RR037Q, Takara, Japan) was used for cDNA synthesis. Real Q Plus 2x and the first strand of cDNA was generated from 500 ng of extracted total RNA using the Takara Prime Script reagent kit according to the protocol provided by the manufacturer. Master Mix Green High ROX (Cat.no: A 325402, Ampliqon, Denmark) was used for real time PCR analysis. SYBER green and ROX were used as the reporter and reference dies, respectively. The relative amount of mRNA for each target was normalized to the gene expression. Gene-specific primer sets used in this study are shown in Table 1. Experiments were repeated three times.

Flow cytometric evaluation

Fibroblasts and endothelial cells were cultured with and without 10% *Aloe vera* for 24 h, and trypsinized cells were transferred into flow cytometry tubes. The cells (10^5) were washed with 1% BSA PBS. The staining of cells was performed with 3 µl of phycoerythrin (PE) labeled antibody (Biolegend, London, UK). The cells were kept in the dark at room temperature for 30 min, and then washed with 1% BSA PBS. The cellular deposition was resuspended in 0.5 ml 1% BSA PBS. Finally, the expression of CD31 was analyzed by winmdi software, and overlaid on unstained cell histograms (36).

Statistical analysis

Results are presented as means ± SEM. Statistical differences between different groups were tested by One-way analysis of variance (ANOVA) using Graph Pad Prism software. A $p < 0.05$ was determined as significant (32).

Table 1. Gene-specific primer sets used for real-time RT-PCR.

ITGA1-F	5'-CGGTACAATCATACAGGCCA-3'
ITGA1-R	5'-TTGCTCCTCCTTCTCTGTTC-3'
ITGB1-F	5'-AATGCCTACTTCTGCACGAT-3'
ITGB1-R	5'-GCTTCTCTGCTGTTCCTTTG-3'
PECAM1-F	5'-CTGGGAGGTCGTCCATGT-3'
PECAM1-R	5'-CACAGGACTCTCGCAATCC-3'

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Results

Normal morphological appearance of fibroblasts and endothelial cells in the presence of *Aloe Vera*

The morphology of fibroblasts and endothelial cells with and without *Aloe vera* was observed using the inverted microscope, and no morphological change was detected in *Aloe vera* treated endothelial cells in comparison with the control group. However, treated fibroblasts appeared more widespread in comparison with the spindle shape of fibroblasts in the control group (Figure 1 A and B). High magnification of fibroblasts by H&E staining showed fan shape morphology of *Aloe vera* treated fibroblasts (Figure 2B). The migration of cells was induced by *Aloe vera* treatment (Figure 2B). There was no obvious change in *Aloe vera* treated endothelial cells at high magnification of H&E staining (Figure 3A and B).

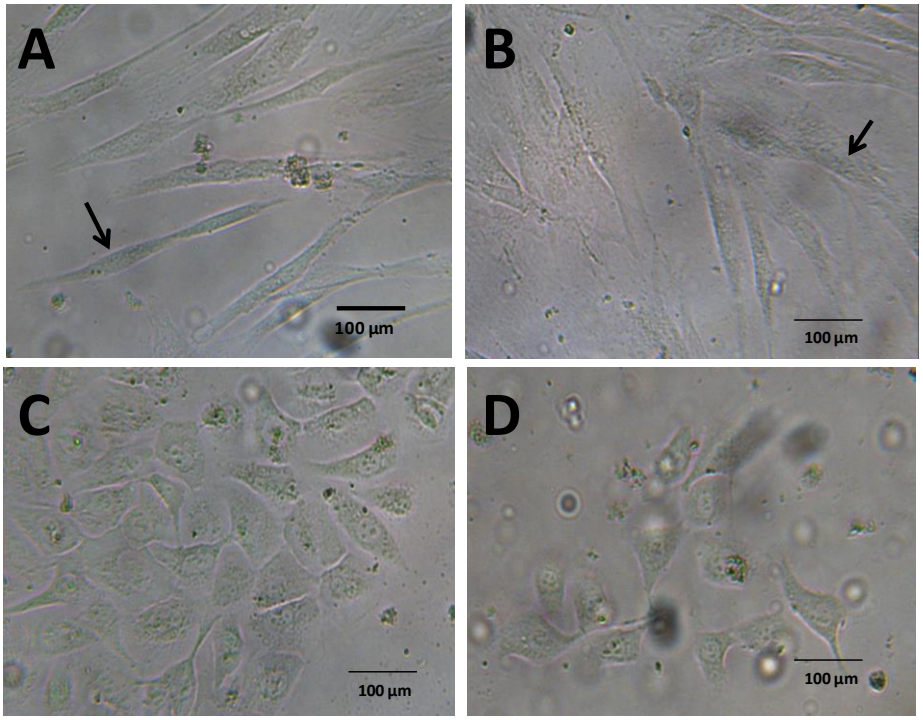

Fig. 1. Morphology of fibroblasts and endothelial cells under inverted microscope. A) Fibroblasts of control group; B) Fibroblasts in the presence of *Aloe vera*; C) Endothelial cells of control group; D) endothelial cells in the presence of *Aloe vera*.

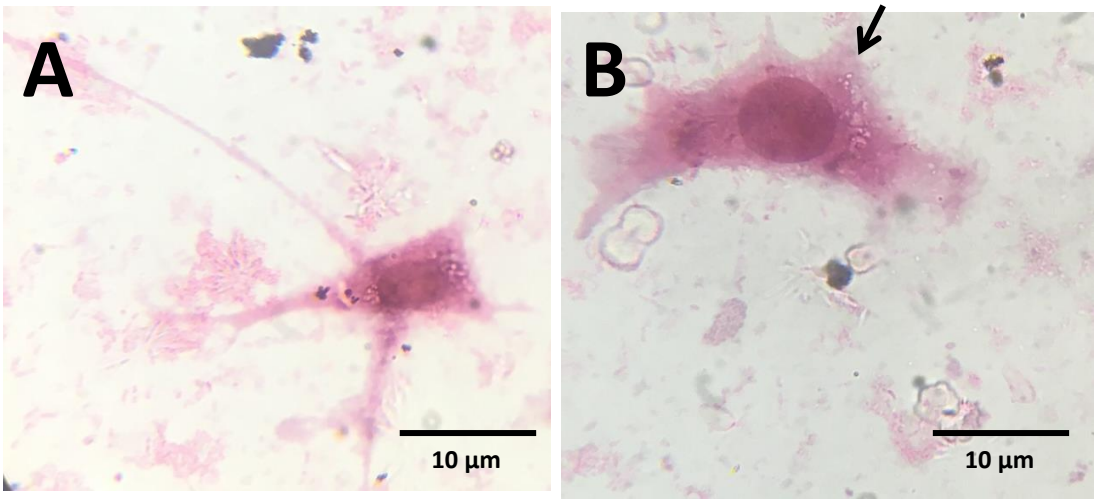

Fig. 2. Morphology of fibroblasts by H&E staining. A) Fibroblasts of control group; B) Fibroblasts in the presence of *Aloe vera*. Migrating fan shape fibroblasts were observed in the presence of *Aloe vera*. Arrow indicates the migrating side of the cell.

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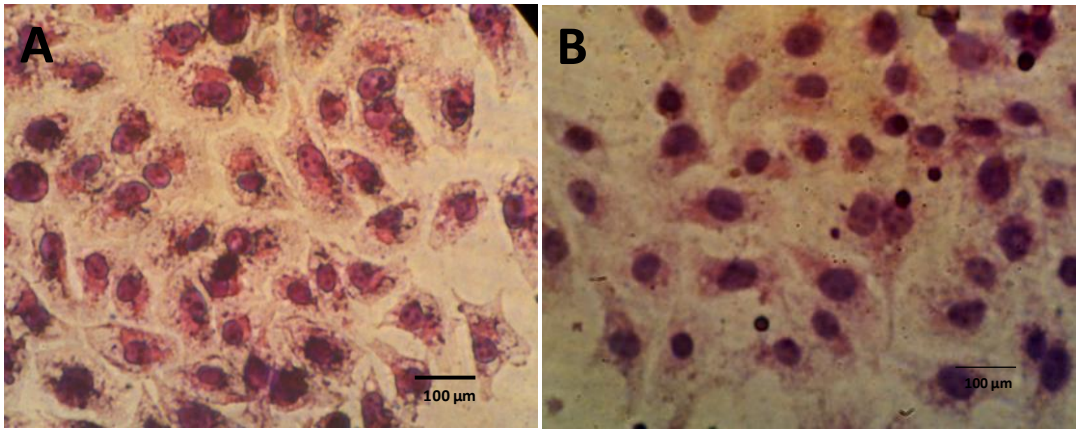
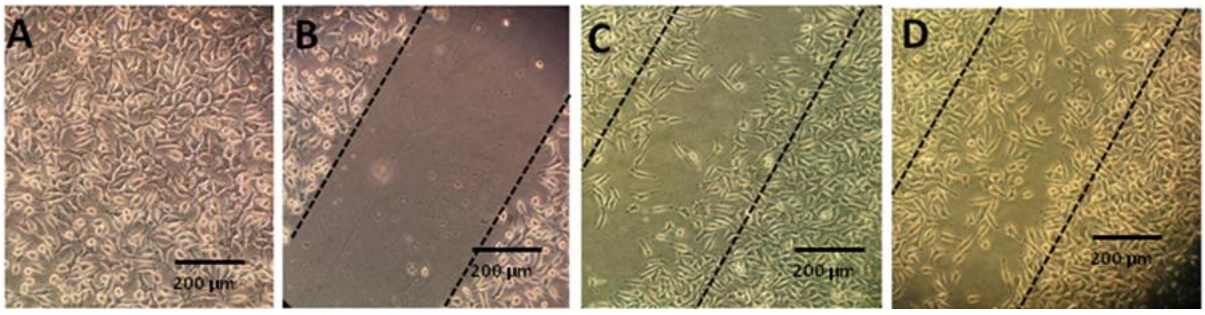


Fig. 3. Morphology **of endothelial cells by H&E staining.** A: Endothelial cells of control group, B: Endothelial cells in the presence of *Aloe vera*.



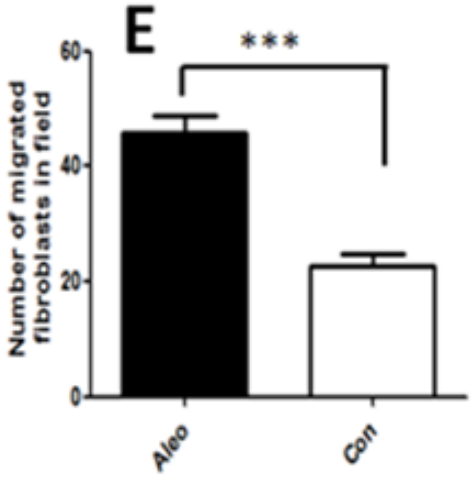


Fig. 4. Scratch assay micrographs. A) Confluent fibroblasts; B) Immediately after scratch; C) Control fibroblasts 24 h after scratch; D) *Aleo vera* treated fibroblasts 24 h after scratch; E) Quantification of migrated fibroblasts to scratch area using image j software (*** P < 0.001).

Aloe vera induced migration of fibroblasts

Our finding showed that fibroblasts proliferated and migrated toward the scratch area during 24 h in the treated group (Figure 4). Statistical analysis revealed that the number of migrated fibroblasts in *Aloe vera* treated groups was significantly (P<0.0005) higher than controls (Figure 4 E)

Aloe vera preserved the viability of fibroblasts and endothelial cells

The toxicity and proliferation rate of *Aloe vera*

[DOI: 10.22088/IJMCM.BUMS.9.3.234]

was evaluated by MTT assay for both cell types. MTT assay revealed that *Aloe vera* has no toxicity on both cell types after 24 and 48 h. Fibroblasts significantly proliferated in the first 24 h but the proliferation rate decreased slightly at 48 h. However, it was still higher in the *Aloe vera* treated group in comparison with the control group. Endothelial cells did not proliferate in the first 24 h, and also the optical density of endothelial *Aloe vera* treated cells significantly (P <0.005) decreased after 48 h (Figure 5).

Aloe vera enhanced the gene expression levels of integrin α1, β1 and _PECAM-1_ (_CD31_), in fibroblasts and endothelial cells

We performed real-time RT PCR analysis to evaluate the gene expression of integrins α1 and β1 to investigate the migrative effect *of Aloe vera* gel. According to the results, *Aloe vera* increased the expression of integrins α_1 and β_1 in fibroblasts and endothelial cells. The ratio of integrins α_1 and β_1 gene expression in fibroblasts containing *Aloe vera* relative to the control group was 19.8 and 2.17, and for endothelial cells was 2.21 and 2.16, respectively (Figure 6 A and B). Therefore, the fold change of integrins α_1 and β_1 gene expressions was significantly (P <0.0001) higher in fibroblasts in comparison with endothelial cells 8.95 and 1.18, respectively (Figure 6 A and B).

Gene expression evaluation of *PECAM-1* in fibroblasts and endothelial cells resulted in significantly higher gene expression in *Aloe vera* treated fibroblasts (P<0.0001) and endothelial cells (P <0.0001) versus controls. Interestingly, *Aloe vera*treated fibroblasts expressed more (P <0.0001) *PECAM-1* gene in comparison with endothelial cells (Figure 6C).

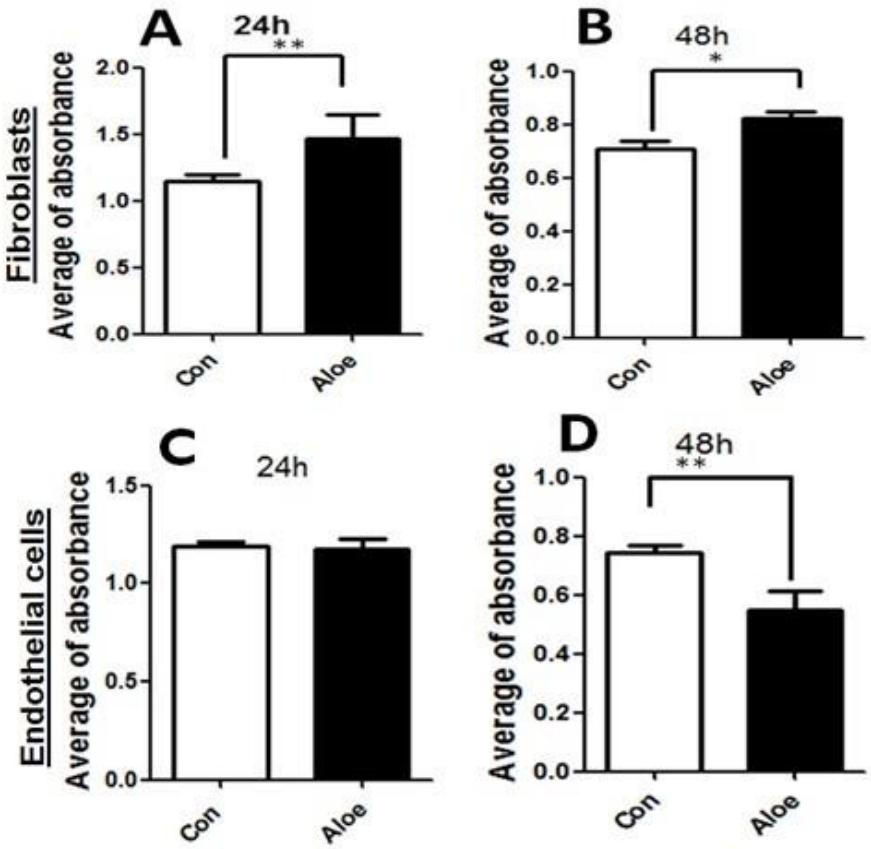


Fig. 5. The effects of _Aloe vera_ gel on fibroblasts and endothelial cells after 24 and 48 h. Cell viability was determined by MTT assay. Cell viability rate in *Aloe vera* groups fibroblasts (A and B) and endothelial cells (C and D) have been compared to controls. Error bars represent the SEM. (* P < 0.05) and (** P < 0.001).

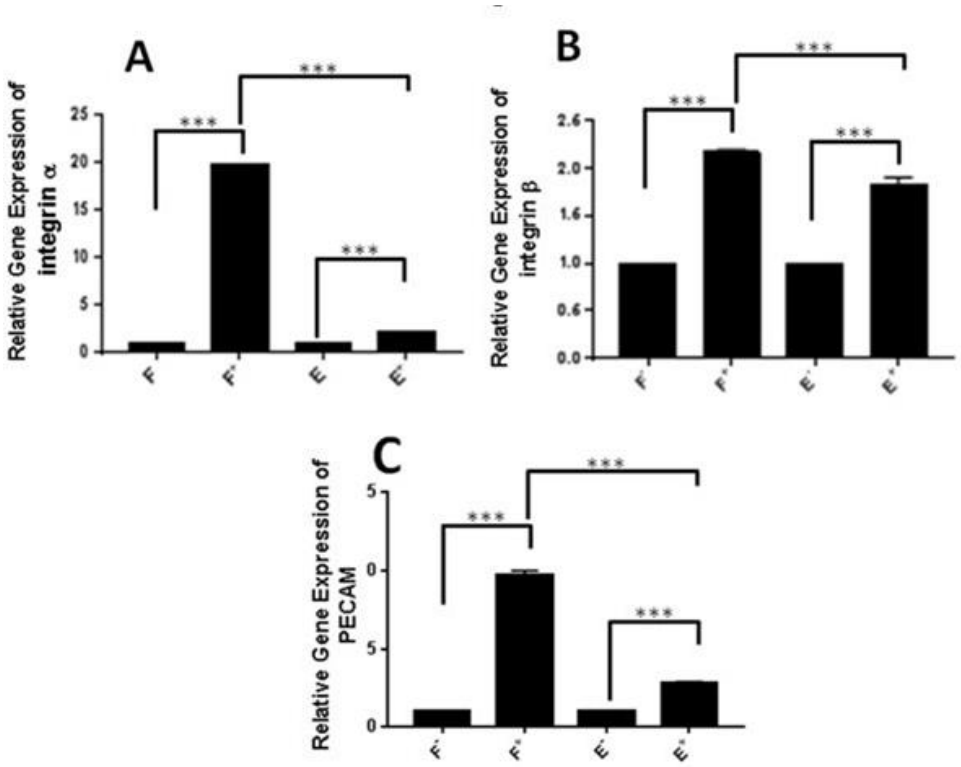


Fig. 6. Relative gene expressions in fibroblasts and endothelial cells by real time RT-PCR. A) integrin α1; B) integrin β1; C: *PECAM-1*. F) Fibroblasts; E) Endothelial cells; cells cultured without *Aloe vera*; +: *Aloe vera* treated cells. Error bars represent SEM (*** P < 0.0005).

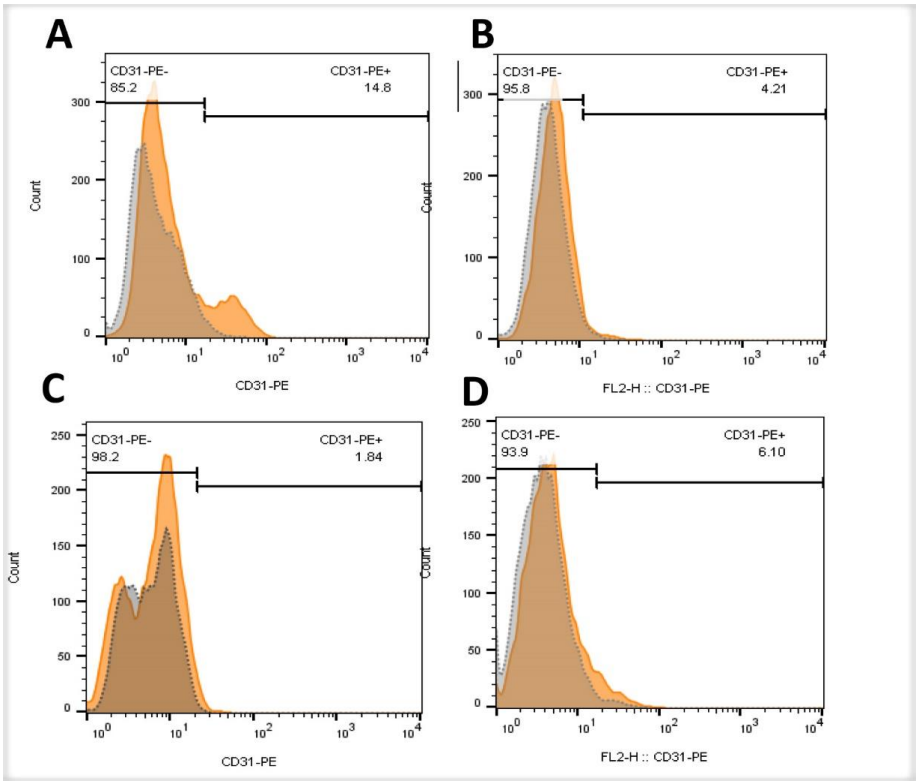


Fig. 7. Immunophenotyping of fibroblasts and endothelial cells for PECAM-1 (CD31). A) fibroblasts of control group; B) fibroblasts in the presence of *Aloe vera*; C) endothelial cells of control group; D) endothelial cells in the presence of *Aloe vera*. Gray histograms indicate unstained cells and orange histograms show CD31-PE stained cells.

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Protein levels of PECAM-1 (CD31) in fibroblasts and endothelial cells by flow cytometry

Flow cytometry of fibroblasts and endothelial cells was performed 48 h after the culture of these cells to examine the expression of CD31 protein. Our data suggested that the expression of CD31 in fibroblasts was 8 times higher than endothelial cells. However, the level of CD31 in *Aloe vera*-treated fibroblasts and endothelial cells had not increased in comparison with their respective control group (Figure 7)

Discussion

This study has compared fibroblast and endothelial cell properties after *Aloe vera* administration. Our findings showed that the morphological changes have occurred in fibroblasts, and *Aloe vera*-treated cells were fan shaped flat cells which are characteristic of migratory cells. These results are in line with our gene expression data that revealed that the expression of integrins α1β1 increased in fibroblasts, but their increase in fibroblasts was significantly higher than endothelial cells. Our findings illustrated that the viability of endothelial cells decreased after 48 h treatment with *Aloe vera* gel. It seems that among the two important cell types of tissue, the wound healing effects of *Aloe vera* is more pronounced in fibroblasts than endothelial cells. However, further *in vivo* studies will be necessary to determine the importance of each cell.

Medicinal plants are frequently used by patients because of their low cost, compatibility with the human body, and fewer side effects (37). *Aloe vera* is one of these herbs that has been used for wound healing from ancient times (38). Several mechanisms such as antioxidant activity (39), increase of matrix metalloproteinases-2 (MMP2), collagen bundles formation (40), and anti-inflammatory process (41) have been suggested for tissue repair properties of *Aloe vera*.

An *in vivo* study demonstrated that oral administration of *Aloe vera* enhanced *TGF-β1* and *VEGF* protein in diabetic rats (42). Also, it has shown that *Aloe vera* induced the expression of *bFGF* and *TGFβ1* in fibroblasts (17). Prakasso showed that the ratio of $CD4^+$ to $CD8^+$ cells increased in the wound area following *Aloe vera* administration, and suggested that the ability of *Aloe vera* in wound healing may be due to an increase in lymphocytes (43). Integrins are key mediators for binding to the ECM. Heterodimers of $\alpha_1\beta_1$ integrins play an important role in cell migration and angiogenesis (44). Beta integrins are highly expressed in fibroblasts (45). Integrins of the β_1 family are involved in wound re-epithelialization (46), myofibroblast differentiation, and in granulation tissue synthesis and remodeling (47). These mechanisms are related to fibroblast function. Cell migration is an integrin mediated multi-stage process that is effective in wound healing (6). Fibroblasts migrate to the wound site 48-72h after injury, and play an important role in all stages of wound healing. In line with our results, Negahdari et al. found that *Aloe vera* can increase fibroblast cell migration and proliferation (48). An *in vitro* study confirmed the above data and showed that *Aloe vera* enhances fibroblast and keratinocyte cell migration and proliferation (49). In this study, we evaluated the gene expression of α_1 and β_1 integrins as factors involved in cell migration in fibroblast and endothelial cells. The expression of both integrins in fibroblast cells was greater than endothelial cells which confirm that the migration of fibroblasts is higher than endothelial cells upon *Aloe vera* gel treatment. Our morphologic and gene expression findings indicated that *Aloe vera* could accelerate wound healing by increasing rather fibroblast cells migration than endothelial cells. Angiogenesis plays a vital role in wound healing process by forming new blood vessels from prior vessels then invading to wound clot and organizing the microvascular network through the granulation

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tissue. This dynamic process is highly regulated by signals from both serum and the surrounding ECM environment (50). Boudreau and Beland indicated that *Aloe vera* gel treatment could shorten wound healing time by increasing angiogenesis (51). Choi et al. found that beta sitosterol of *Aloe vera* enhanced angiogenesis by increasing *VEGF* gene expression in chick embryo chorioallantoic membrane assay (52). Aloesin an anthraquinone of *Aloe vera* leaves induced angiogenesis via activation of SMAD and MAPK signaling proteins in endothelial cells (53). An *in vivo* study confirmed the above data and demonstrated that oral administration of *Aloe vera* enhanced Tgf-β1 and Vegf protein in diabetic rats (42). *Aloe vera* gel could up-regulate *Tgfβ1* gene expression with a dose-dependent and time-dependent manner in mouse embryonic fibroblast cells as well (17).

In 1997, DeLisser et al. introduced PECAM-1 as an angiogenic factor (54). The absence of PECAM-1 in endothelial cells results in the increase of cell motility and poor migration in wound healing (3). Our previous study demonstrated that *Aloe vera*/collagen blended can be useful in the tissue engineering process by enhancing *PECAM-1* gene expression in adipose-derived stem cells (32). *PECAM-1* was upregulated by *Aloe vera* gel in fibroblasts. However, flow cytometric data were controversial probably due to undetectable protein expression level during 48 h treatment with *Aloe vera*. It seems that longer treatment time should be investigated to evaluate the post-translational regulation of PECAM-1

In general, the effect of *Aloe vera* on proliferation, angiogenesis, and cell migration in fibroblasts seems to be more than endothelial cells. Therefore, the wound healing effect of *Aloe vera* was probably due to the effect of this plant on fibroblast cells. We concluded that *Aloe vera* could accelerate wound healing by increasing α1β1 integrins and *PECEM-1* expression. However, more investigations are needed to show the main role of fibroblasts in wound healing in comparison with endothelial cells by *Aloe vera* administration.

Acknowledgement

We would like to thank the Zanjan University of Medical Sciences for funding this research and their great support in study development. The eEthics Committee of Zanjan University of Medical Sciences approved the study protocol with code number 243.

Conflict of interest

No conflict of interest in terms of scientific collaboration and financial benefits is declared by the authors of the study.

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[DOI: 10.22088/IJMCM.BUMS.9.3.234]