Biomedicine & Pharmacotherapy 88 (2017) 610–616

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Original article

The effect of aloe vera on the expression of wound healing factors (TGFβ1 and bFGF) in mouse embryonic fibroblast cell: In vitro study

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ARTICLE INFO

Article history:
Received 23 October 2016
Received in revised form 11 January 2017
Accepted 16 January 2017

Keywords:
Fibroblast
Aloe vera (A.v)
bFGF
TGFβ1

ABSTRACT

Background: Aloe vera (A.v) have been used traditionally for topical treatment of wounds and burns in different countries for centuries, but the mechanism of this effect is not well understood. Various growth factors are implicated in the process of wound healing. Among the different growth factors involved in the process, TGFβ1 and bFGF are the most importantly expressed in fibroblast cells. The aim of this study was to evaluate the effect of A.v on the expression of angiogenesis growth factors in mouse embryonic fibroblast cells.

Methods: We exposed mouse embryonic fibroblast cells to different concentrations of A.v (50, 100 and 150 μg/ml) at two different time of 12 and 24 h. Fibroblast cell without A.v treatment serves as the control. The expression of TGFβ1and bFGF was measured by real time-polymerase chain reaction (real-time-PCR) and enzyme-linked immunosorbent assay (ELISA) at the level of gene and protein.

Results: We observed that A.v gel at first up-regulated the expression of TGFβ1 and bFGF, but, these genes were later repressed after a particular time.

Conclusion: Our results demonstrated that A.v was dose-dependent and time-dependent on the expression of bFGF and TGFβ1 in fibroblast cell in vitro. This mechanism can be employed in the prospective treatment of physical lesion.

1. Introduction

Wound healing is a highly complicated process, comprising of cascade of healing events. The normal process of wound repair consists of four overlapping phases with a predictable series of biochemical and cellular event; hemostasis, inflammation, tissue formation (the proliferative phase), and finally tissue remodeling [1–4]. These processes are affected by several factors such as cytokines, growth factors and low-weight molecular compounds. Angiogenesis is important in many processes such as wound healing, kidney function, fetal development, reproduction and fertility preservation [5,6]. Wound healing is a response to injured tissue, resulting in the restoration of tissue integrity.

Several investigations show that growth factors play a crucial role in cell division, migration, differentiation, enzyme production, and protein expression. These factors can be responsible for wound healing through the stimulation of angiogenesis and cellular proliferation. This in-turn affects both the production and degradation of the extracellular matrix (ECM), and chemotactic for the recruitment of inflammatory cells and fibroblasts [7].

Two key growth factors are implicated in the process of wound healing, these are basic fibroblast growth factor (bFGF) and transforming growth factor β1(TGFβ1) [8]. The later control proliferation of fibroblast, transformation into myofibroblasts, the production of ECM, stimulation of collagen production, elastin production, and fibronectin synthesis while inhibiting ECM degradation [9]. bFGF is also implicated in the process of wound healing. It is capable of regulating the replication and migration of epithelial, endothelial, and fibroblasts cells, which partake in collagen production, epithelialization and neovascularization respectively [10].

The A.v plant is known as “the healing plant”. This plant has been traditionally used to treat wounds and burns [11]. The oral and topical administration of Av gel has been reported to be effective both in normal and diabetic wounds [12,13]. It contains several medicinal properties such as wound healing, promotion of radiation damage repair, anti-inflammatory effects, anti-bacterial, anti-viral, anti-fungal, anti-diabetic and antineoplastic activities,

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http://dx.doi.org/10.1016/j.biopha.2017.01.095
0753-3322/

immunostimulation stimulation of hematopoiesis and anti-oxidant effects [14]. Studies suggest that treatment with either A.v crude gel or its extracted components like β-sitosterol, acemannan, etc, resulted in faster wound healing through the stimulation of growth factor production, angiogenesis, proliferation of fibroblast and collagen deposition and production of growth factors [3,15]. A.v gel was shown to ameliorate wound healing after systemic and topical administration in several studies, in spite of all the studies on A.v, the mechanism by which it carries out its effect is yet to be understood [16].

Previous in vitro study by Jettanacheawchankit et al. was carried out on acemannan (polysaccharide from A.v), to demonstrate its effect on type I collagen, keratinocyte growth factor-1 (KGF-1) and VEGF production [17]. In vivo investigations using A.v treatment on wound healing mechanism has been carried out as well. A.v β-sitosterol improves angiogenesis triggered by VEGF, blood vessel matrix laminin, Von Willebrand factors and VEGF receptor [18,19]. Yet, these studies was carried out on chick embryo chorioallantoic membrane assay. In addition to this, another study by Atiba et al. [20] have demonstrated the effect of oral administration of A.v on cutaneous wound healing, analyzing VEGF and TGF-1 β expression, in a type 2 diabetic rat model. This was an *in vivo* study as well and bFGF expression was not determined. In our study, we aim at investigating the effect of A.v on the expression of bFGF and TGFβ1 in mouse embryonic fibroblast cells at the level of gene and protein in vitro.

2. Material and methods

The study was designed to compare TGFβ1 and bFGF expression in mouse embryonic fibroblasts cells, treated by different concentrations of A.v. The concentration of our extract in these study are (50, 100 and 150 μg/ml), and the control of our study is the fibroblast cell without the active ingredient at (12 and 24 h after treatment).

2.1. A.v gel separation

Freshly harvested A.v leaves were thoroughly washed with sterile water, and its skin was peeled off using a sterile condition. The inner gel was collected and frozen at −80 °C, and was subsequently lyophilized and stored at −20 °C until further use.

2.2. Isolation and culture of mouse embryonic fibroblasts (MEFs)

In order to isolated the mouse embryonic fibroblast, we performed the following steps according to the protocol of Jozefczuk et al. [21]. The first step was to anesthetize and sacrifice a pregnant mouse at 13 or 14 d.p.c (day post-coitum) by dislocating of cervical vertebrae. After that, the uterine horns was dissected out, briefly rinsed in 70% (v/v) ethanol and placed in a falcon tube along with the buffer PBS without calcium and magnesium ions. The following steps were performed under aseptic conditions with sterile instruments in a tissue culture hood. First, the uterine horns was placed in petri dish, and each its embryo was separated from its embryonic sac and placenta. Head and the red organs were dissected and washed in PBS buffer, then, all embryos were placed in a sterile petri dish. The tissue is then minced using an intact razor blade until we could pipette it. Then, 1 ml of 0.05% trpsin/EDTA (Gibco, Invitrogen) containing 100 K units of DNase I per each embryo was added. After that, the tissues were transferred to a 50 ml falcon tube, incubated for 15 min at the room temperature. Following each 5 min of incubation the cells were dissociated by pipetting thoroughly. In the next stage, trypsin was inactivated through the addition of 1 vol of freshly prepared MEF medium; a culture medium (components to make 500 ml of media, all components and filters was mixed) which consists of 450 ml of DMEM, 50 ml of FBS (10% (v/v)), 5 ml of Penicillin-streptomycin (1/100 (v/v)) and 5 ml of 200 mM L-glutamine (1/100 (v/v)). The cells are centrifuged with low speed (300 × *g*) for 5 min. Then the supernatant was discarded, and cell pellet was in warm MEF medium. After that, we plated approximately a number of cells equivalent to 3–4 embryos in each T150 (TPP) flask which was coated with 0.2% of bovine gelatin (Gelatin from bovine skin, type B, Sigma) for 2 h. At this time, the fibroblasts (P0, passage 0) were the only cells which were able to adhere to the gelatin-coated flasks. Ideally, the cells were confluent about 80–90% after 24 h and at this level, a majority of P0 cells were frozen for future usage. At the end of the procedure, the remaining T150 flasks P0 cells were stored for further study.

2.3. MTT assay for cell viability determination

Colorimetric MTT assay was performed to assess cell viability. Briefly, 100 μl/well fibroblasts cells (10^4 cell per each well) were added into 96-well plates and allowed to adhere for 24 h. It was incubated with different concentrations of A.v in water medium (0, 50, 100 and 150 μg/ml) for 24, 48 and 72 h. At the end of the treatment, 20 μl of MTT (5 mg/ml, Sigma) in PBS solution was added into each of the well, and then the plate was further incubated for 4 h. The remaining supernatants were removed and 200 μl of DMSO was added into each well and thoroughly mixed to dissolve the formed crystal formazan. After incubating for 15 min to ensure all crystals have been dissolved, the light absorption was measured by using an enzyme-linked immunosorbent assay (ELISA) reader. Viability was expressed as a percentage of absorbance values in treated cells to that in control cells.

2.4. Analysis of TGFβ1 and bFGF gene expression by real time-PCR

Mouse embryonic fibroblast cells were grown in 6-well plates (10^5 cell per each well). Cells were exposed to different concentrations of A.v (0, 50, 100 and 150 μg/ml) and cells were collected at 12 and 24 h by process of trypsin/EDTA. Total RNA was isolated from the cells using the Total RNA Purification Kit (Jena Bioscience, Germany) according to the instruction of the manufacturer's. All RNA preparation and handling steps were done under RNAse-free conditions. The concentration and purity of RNA was determined using biophotometer (Eppendorf, Hamburg, Germany). The concentration and quality of the RNA samples were later confirmed by electrophoresis on denaturated 1% agarose gel. cDNA was synthesized from 1 μg total RNA using the cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). The house keeping gene for normalization was HPRT. The oligonucleotide sequences of the primers are presented in Table 1.

Real-time quantitative PCR was performed with Rotor-Gene 6000 real time PCR system and SYBR-Green quantitative PCR (qPCR) kit (Jena Bioscience). The qPCR reaction was prepared in a total volume of 20 μl containing 10 μl of 2X SYBR Green master

Table 1
Sequences of primers for Real-time quantitative PCR.

Gene	Primer	Product size	Tm
HPRT	Sense:CCTCCTCAGACCGCTTTTT Antisense:AACCTGGTTCATCATCGCTAA	91	79.5
FGF2	Sense: AACGGCGGCTTCTTCCTG Antisense:TGGCACACACTCCCTTGATAG	133	78.9
TGFβ1	Sense: ATTCCTGGCGTTACCTTGG Antisense:CCTGTATTCCGTCTCCTTGG	117	76.9

mix, 1.6 μl of the cDNA template, 0.6 μl of each primer (10 pmol/μl) and 7.2 μl of deionized water. A negative control was used by replacing the cDNA template with deionized water. Primer sequences used in this study and their annealing temperature are shown in Table 1. The PCR amplification started with initial denaturation and polymerase activation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 40 s and extension at 72 °C for 30 s. The specificity of PCR products was verified by melting curves and electrophoresis through 1% agarose gel.

2.5. Analysis of TGFβ1 and bFGF protein expression by ELISA

Mouse embryonic fibroblast cells were grown in 6-well plates (10^5 cell per each well), cells were exposed to A.v at 0, 50, 100 and 150 μg/ml, and its supernatant were collected at 12 and 24 h. Samples were freezed at −20 °C. TGF-β1 and bFGF levels from cell culture supernatants were measured by ELISA kit (TGF-β1 ELISA kit, eBiosciences, bFGF ELISA kit, RayBiotech, Inc) according to the instruction of the manufacturer's.

To measure TGF-β1 levels, the Ready-Set-GO TGF-β1 cytokine ELISA kit from eBioscience (San Diego, CA, USA) Human/Mouse TGF beta1 ELISA Ready-SET-Go (Catalog Number: 88–8350) were utilized. Briefly, it was filled on a 96-well plates overnight with a suitable dilution for capturing antibody in 0.2 M sodium phosphate buffer with pH 6.5. The plate was washed tree times with PBS plus 0.05% Tween-20 (PBS-T), and then blocked with Assay Diluent at 21 °C for 1 h. In order to inactivate latent TGF-β1, the samples were treated with 20 μl solution of 1 N HCl per 100 μl of sample for 10 min and after that neutralized with 1 N NaOH. Then, 100 μl of sample or cytokines prepared in assay diluent were added and incubated at 21 °C for 2 h. After five times of washing with PBS-T, 100 μl of assay diluent containing biotin-conjugated detecting antibody and avidin-horse radish peroxidase reagent at the suitable dilutions were added and incubated at 21 °C for 1 h. After seven times of washing with PBS-T, 100 μl of the substrate solution was added to each well plate. Following 30 min of incubation in dark condition, 50 μl of the stop solution (2 N H_2SO_4) were added. The ELISA result were read within 30 min by using an enzyme-linked immunosorbent assay (ELISA) reader. The final calculation was performed using a dilution factor of 1.4 in order to investigate acid activation/neutralization.

Mouse bFGF release in the supernatant was detected with mouse basic FGF ELISA kit (Cat no.ELH-bFGF-001; RayBiotech, Inc., St. Louis, MO). As a brief description, 100 μl were added to each cell culture supernatant into appropriate wells. The wells were covered and incubated at room temperature for 2.5 h. Then, the solution was discarded and washed 4 times with 200 μl of wash solution (1×) for each well. Then, 100 μl of 1x prepared biotinylated antibody were added into each well. The plate was incubated at the room temperature for one hour. Repeatedly, the solution was discarded and washed 4 times with 1 × 200 μl of wash solution for each well. After the washing, we added 100 μl of prepared streptavidin solution into each well and waited for 45 min for incubation at the room temperature. The solution was discarded once again, and washed for 5 times with 1 × 200 μl of sash solution for each well. Then after, we added 100 μl of TMB One-Step Substrate Reagent to each well and incubated at the room temperature for 30 min in dark condition. Finally we added 50 μl of stop solution and read immediately at the absorbance 450 nm. The results were expressed as bFGF level in pg per ml of cell culture supernatant.

2.6. Blocking of TGF β1 activity

To systemically inhibit TGF β 1 function, samples from analysis of TGFβ1 gene and protein expression by Real Time-PCR. They were treated using a monoclonal antibody (mab) against mouse TGF β 1 (TGFmab; 5 mg/kg diluted in 150 μl of PBS), clone 1D11 (Bio-x-cell, West Lebanon, NH) after 24 h of A.v administration. This antibody has been employed in several studies and has been shown to be efficient in blocking signaling in vivo of all TGF β 1 isoforms [22]. The sample in the control group were treated with a similar dose of isotype control mab. The expression of TGF β 1 protein was re-analysed according to the previous protocol.

2.7. Statistical analysis

Our result are from different independent experiments, the total expression ratio of the genes of interest at three different concentrations was compared between Av and control groups using a randomization test implemented in the relative expression software tool (REST), which is an Excel-based application for comparison and statistical analysis of relative expression results in qRT-PCR [23]. Protein expression in the two groups were compared

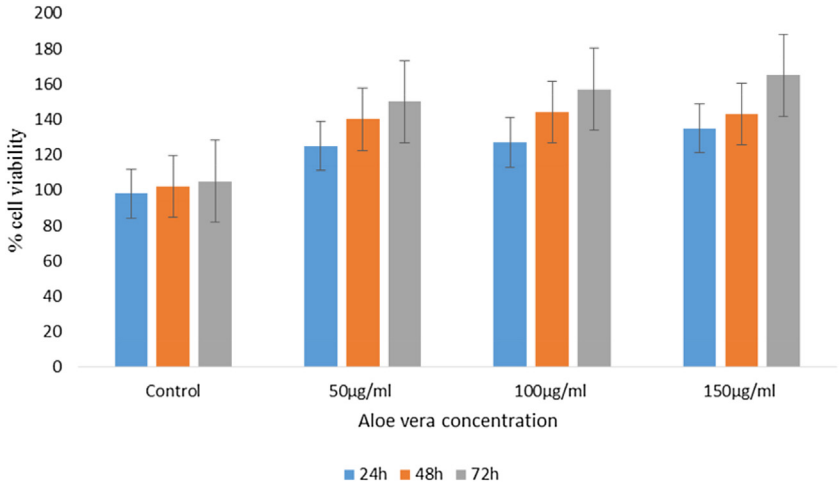

Fig. 1. Effect of various concentrations of A.v on mouse embryonic fibroblast cell viability. Cells were treated with different concentration of A.v for 24, 48 and 72 h and cell viability was measured by MTT assay.

by the Statistical Package for the Social Science (SPSS, Cary, NC). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effect of A.v on mouse embryonic fibroblast cell viability

The results of MTT assay showed that A.v gel was non-toxic to the cells (Fig. 1). Some studies have showed that A.v had no toxic effect on macrophages and peripheral blood mononuclear cells up to a concentration of 100 μg/ml.

3.2. TGFβ1 and bFGF gene expression

Analysis of TGFβ1 gene expression by real-time RT-PCR showed an opposite patterns at the two study time. There was no significant difference between 0 μg/ml and 50 μg/ml at both time point, however, there was a significant increase in this gene at different concentrations of 100 and 150 μg/ml at 12 h after treatment by A.v as compared to the control ($P = 0.001$, $P = 0.001$ and $P = 0.001$ respectively). But the expression of TGFβ1 gene decreased at 24 h after treatment which was significant at a concentration of 150 μg/ml (Fig. 2, Table 2), showing no significant difference at every point.

Gene expression of bFGF in cells treated with A.v showed similar patterns with TGFβ1 gene expression. There was no significant difference between 0 μg/ml and 50 μg/ml at both time point, however, there was a significant increase in this gene at different concentrations of 100 and 150 μg/ml at 12 h after treatment by A.v as compared to the control ($P = 0.001$, $P = 0.001$ and $P = 0.001$ respectively). But the expression of bFGF gene decreased at 24 h after treatment which was significant at a concentration of 150 μg/ml, showing no significant difference at every point (Fig. 3, Table 3).

3.3. TGFβ1 and bFGF protein expression

The pattern of TGFβ1 and bFGF protein expression, were similar to those of the genes expression, but no strict consistency in gene expression. The increase in TGFβ1 protein expression was significant at the three concentrations studied at 12 h, but the decrease in protein expression 24 h after treatment was significant at 100 and 150 μg/ml (Fig. 4). bFGF protein expression was increased significantly 12 h after treatment at all concentrations of study but decrease of protein expression 24 h after treatment was significant at all concentrations of study (Fig. 5).

3.4. TGF β1 blocking and expression

The blocking of TGF β1 for gene and protein expression at 24 h was shown in (Fig. 6), it was able to inhibit overproduction. From the graph, it was observed that, the antibody blocking was able to reverse the inhibition of the expression at 24 h which was significant at the concentrations studied. The decrease of TGF β1 at 24 h was due to feedback mechanism ($P = 0.001$).

4. Discussion

The present investigation shows the effect of A.v on the expression of bFGF and TGFβ1 in mouse embryonic fibroblast cells at the level of gene and protein in vitro, which can be as a result of improved fibroblasts and endothelial cells stimulated by bFGF and TGF-β1 migration.

Previous studies suggests that A.v active components like acemannan can bind special ligands to mannose receptors presented on the cell surface of fibroblasts and macrophages, this binding results in cells triggering the growth factors and proliferation [24].

In the present study, MTT analysis of A.v showed that the plant was not toxic at the analysed concentrations in the study, this result is similar to that reviewed by Reynolds et al., [25] and research study by Gopakumar et al., [26].

The main finding of our study was that A.v gel at first (12 h) up-regulated the expression of TGFβ1 and bFGF, but after some time (24 h), the expression of these genes were down-regulated. Analysis of gene and protein expression by real-time RT-PCR showed a significantly increases at different concentrations for the first 12 h after treatment with A.v as compared with the control. However, these expression was decreased 24 h after treatment, which was significant at some concentrations of treatment with A. v for both TGFβ1 and bFGF. The mechanism of by which A.v stimulates the production of growth factor and fibroblast is still unclear. However, in this study, the down-regulation of TGFβ1 at 24 h might be due to feedback mechanism inhibiting

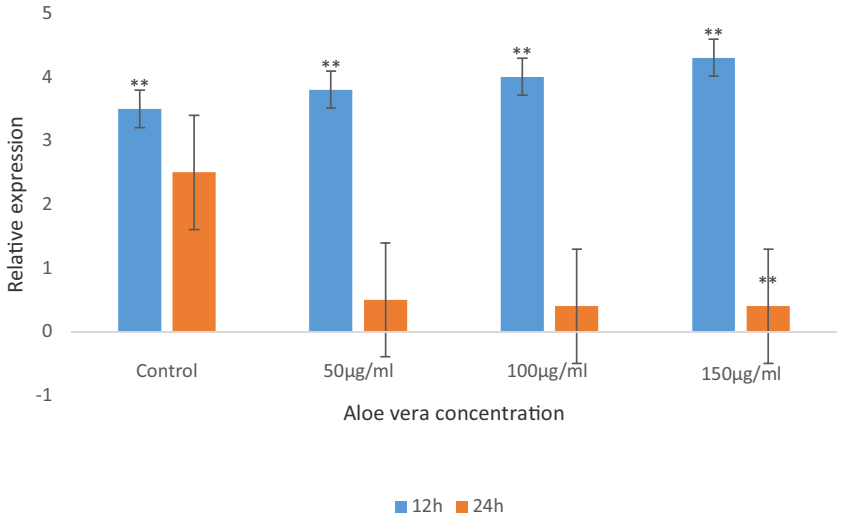

Fig. 2. Relative expression of TGFβ1 gene in mouse embryonic fibroblast cell, treated with various concentrations of A.v as compared with the control group at different time intervals (12 and 24 h). Mouse embryonic fibroblast cells were treated with A.v extracts (50, 100 and 150 μg/ml) and the expression of TGFβ1 gene was assessed by quantitative real time PCR. All comparisons analysed with the control group. $P < 0.05$, $P < 0.01$.

Table 2
The effect of different concentrations of Aloe vera on expression of TGFβ1 gene.

	12 h after treatment			24 h after treatment		
	10 μg/ml	20 μg/ml	30 μg/ml	10 μg/ml	20 μg/ml	30 μg/ml
Relative expression	2.72	2.84	3.12	1.36	0.70	0.64
Standard error	±0.48	±0.53	±0.67	±0.36	±0.26	±0.17
P-value	0.001	0.001	0.001	0.32	0.32	0.001
Fold increase/decrease	+2.72	+2.84	+3.12	+1.36	−1.43	−1.56

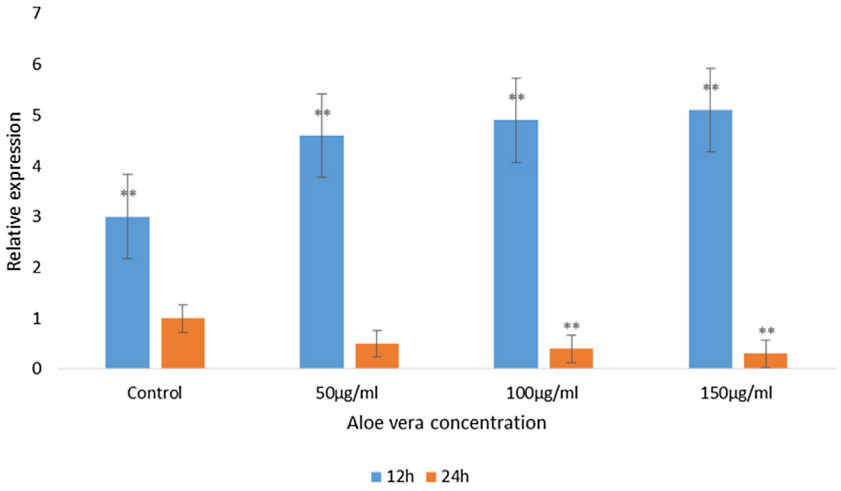

Fig. 3. Relative expression of bFGF gene in mouse embryonic fibroblast cells treated with various concentrations of A.v as compared with the control group, at different time intervals (12 and 24 h). Mouse embryonic fibroblast cells were treated with A.v (50, 100 and 150 μg/ml) and the expression of bFGF gene was assessed by quantitative real time PCR. All comparisons were made with the control group. $P < 0.05$, $P < 0.01$.

Table 3
The effect of different concentrations of Aloe vera on expression of bFGF gene.

	12 h after treatment			24 h after treatment		
	10 μg/ml	20 μg/ml	30 μg/ml	10 μg/ml	20 μg/ml	30 μg/ml
Relative expression	3.12	3.96	4.46	0.64	0.50	0.45
Standard error	±0.45	±0.53	±0.68	±0.21	±0.17	±0.18
P-value	0.001	0.001	0.001	0.32	0.001	0.001
Fold increase/decrease	+3.12	+3.96	+4.46	−1.56	−1.98	−2.23

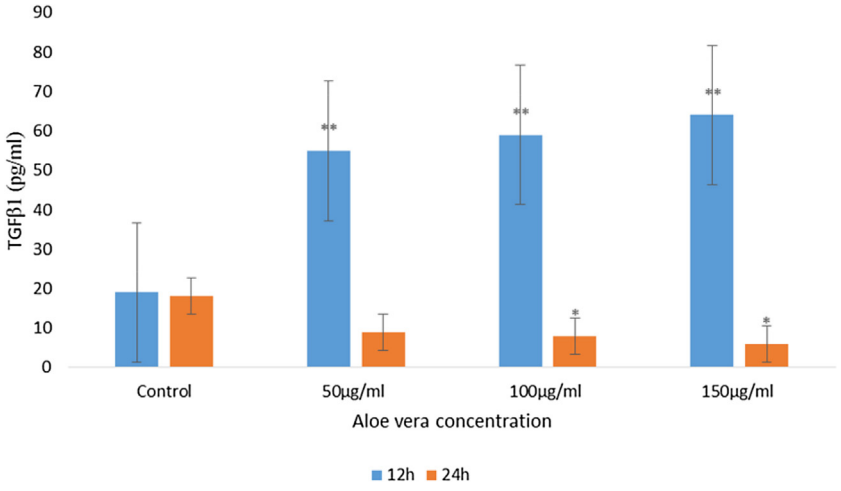

Fig. 4. Effect of various concentrations of A.v on the expression of TGFβ1 protein in mouse embryonic fibroblast cell culture supernatants. Cells were treated with different concentration of A.v (50, 100 and 150 μg/ml) at different time intervals treatment (12 and 24 h) and the expression of TGFβ1 protein was assessed by ELISA. $P < 0.05$, $P < 0.01$.

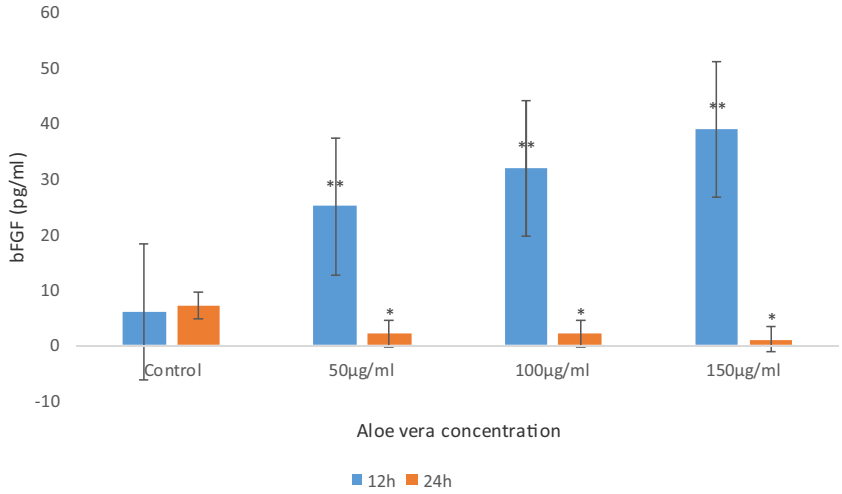

Fig. 5. Effect of various concentrations of A.v on the expression of bFGF protein in mouse embryonic fibroblast cell culture supernatants. Cells were treated with different concentration of A.v (50, 100 and 150 µg/ml) at different time intervals treatment (12 and 24 h) and the expression of bFGF protein was assessed by ELISA. $P < 0.05$, $P < 0.01$.

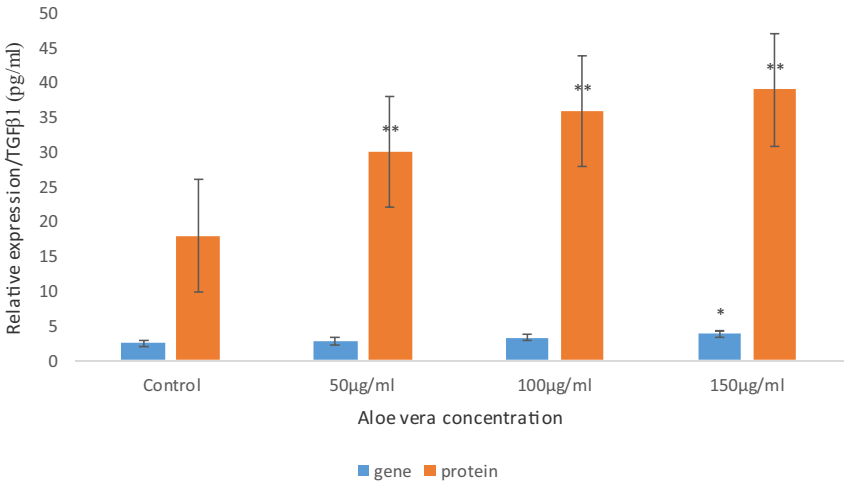

Fig. 6. The effect of blocking TGFβ1 gene and protein 24hrs at the different concentration studied. After the addition of anti-TGFβ1 antibody, there was a sharp expression of the gene particularly at the highest concentration of 150 Ug/ml. $P < 0.05$, $P < 0.01$.

overproduction of these factors, and the result of our experiment in Fig. 6 shows that, blocking of TGFβ1 over-production reversed the inhibition and shows a sharp expression of the gene particularly at the highest concentration.

Despite the important role of fibroblasts, TGFβ1 and bFGF in wound healing, it has been shown that the up-regulation of TGF-β1 consistently lead to fibrotic disease, blocking its bioactivity may inhibit the production of matrix and modulate the fibrotic process [27]. TGFβ1 does not only increase transcription of collagen, but also decreases its degradation through inhibition of collagen activity by increasing production of MMP inhibitor like plasminogen activator inhibitor and TIMP. A similar results were also obtained from other studies in human peritoneal fibroblasts [28].

The results of this study showed that A. Vera can increase the production of TGF β1 and bFGF, which will stimulate the collagen deposition, fibroblast proliferation, and angiogenesis, but after that, down-regulation of these factors can inhibit overproduction and accumulation of matrix proteins which cause hypertrophic scar.

5. Conclusion

Our study demonstrated A.v would provide a systemic effect on fibroblast cells in vitro via growth factors production and angiogenesis. Our investigation opens an avenue for prospective research and application of this plant extract on ameliorating delayed wound healing problems. However, further study still is required to confirm this speculation.

Conflict of interest

There is no conflict of interest in this paper.

Acknowledgment

This work was supported by a grant from Lorestan University of Medical Science, Korramabad, Iran with grant number 100/394242.

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