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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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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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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