

Galectin 1 and Superoxide Dismutase are Involved in Wound Healing by Larval Therapy

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ABSTRACT

Galectin-1 and superoxide dismutase are two known molecules in the wound healing process that induce such healing by different mechanisms in the wound site. Larval therapy is one of the methods by *Lucilia sericata* fly larvae, nowadays returned to the list of therapeutic methods despite chronic diabetic ulcers and antibiotic resistance of bacteria. In this study, we aimed to evaluate the effect of larvae extract on fibroblast cells to determine its role in the levels of Galectin-1 and superoxide dismutase proteins in fibroblast cells. To determine proteomic changes, 3T3 fibroblast cells were treated with larval extract, 3T3 fibroblast cells were cultured and divided into two groups after appropriate density. The first group was considered as control and the second group was treated with larvae extract at a concentration of 12.5 µg/ml. After 24 hours, the two-dimensional gel electrophoresis method for protein level and real-time PCR for gene expression studies were used. In 2D gel testing, three spots were successfully identified including galectin-1, superoxide dismutase, and glyceraldehyde-3-phosphate dehydrogenase. The expression of these three proteins was significantly increased in the cells treated with larvae extract compared to the control cells. Also, the quantitative expression of these genes was confirmed by real-time PCR. Finally, it was found that the treatment of fibroblast cells with larvae extract increased expression of galectin-1, superoxide dismutase and glyceraldehyde-3-phosphate dehydrogenase, which their positive effect on wound healing is well known.

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Introduction

Today wound management and treatment, a significant portion of health care are dedicated, and proper wound management is one of the key determinants of medical success in the world (Myers, 2008; Rezaie *et al.*, 2019). Although modern techniques and methods have often been developed in the field of wound healing based on advances in the production of synthetic drugs, the microbial resistance to antibiotics and synthetic drugs in recent years, as well as the growing rate of chronic ulcers, have led to the inefficiency of current wound treatments especially in the case of chronic wounds (Schultz *et al.*, 2003). One method that has been focused

in the last two decades is the treatment of wound using *Lucilia sericata* larvae which called maggot therapy (Sanjari *et al.*, 2013). The mechanisms of larval therapy include debridement, disinfection, and growth stimulation (Sherman, 2002). Not all species of flies are suitable for this aim, it's just *Lucilia sericata* larvae, which have been disinfected by specific techniques, are used for treatment (Davydov, 2011). Until yet Some research has been carried out on the effect of *Lucilia sericata* larvae extract on fibroblast proliferation and migration with hyaluronic/collagen membrane and have provided some evidence that proved Larval secretions contain certain proteinases, including proteases that are involved in the



repair of the extracellular matrix (ECM) that able larvae to wound debridement (Sherman, 2002). The amount of larvae required for wound healing depends on factors such as the amount of necrotic tissue, the extent of the wound, the depth of the wound and the bacterial species in the case of infection (Church, 1999). Larvae are more effective on Gram-positive bacteria than Gram-negative bacteria, and if the wound is infected with Gram-negative bacteria, more larvae will need to wound healing (Kaihanfar *et al.*, 2018). Larvae can be used to treat chronic ulcers from diabetes and this is a good way to treat elderly people who have more problems and incur high costs for treating non-healing wounds (Sherman, 2002). Today many countries and clinics use this treatment method. There have been no reports of lethal effects on patients' lives and health so far, but they can also have several side effects including pain, bleeding, and swelling of the skin, fever which can be managed and controlled by a physician. Wound debridement is defined as the removal of necrotic tissue, foreign substances, and also removing bacteria, the presence of which inhibits the body's normal healing (Boateng *et al.*, 2008). The larvae can swallow the bacteria (Daeschlein *et al.*, 2007) and effectively prevent the formation of biofilm by the bacteria (Jaklič *et al.*, 2008). In general, larval therapy is a simple, safe, effective and low-cost method of wound healing (Kaihanfar *et al.*, 2018). Galectin-1 gene acts as a key element in wound healing. It generates galectin-1 protein, which can affect the fibroblasts migration and function in the extracellular matrix production in the wound area and thus speed up wound healing (Lin *et al.*, 2015).

The healing role of superoxide dismutase in wound healing is well known to inhibit ROS at the wound site and help wound healing Marrotte *et al.*, 2010). Studies have also shown that this protein plays an effective role in wound healing by reducing the extent of the wound and preventing edema formation (Vorauer-Uhl *et al.*, 2001).

In a previous study, we well established the role of larvae extract on wound healing and the inhibitory effect of the extract on the pathogenic microbes (Sanjari *et al.*, 2013). The current study aimed to investigate the effective molecules in

fibroblast cells triggered by the larval extract. This study aimed to investigate the molecular and cellular mechanism of the effect of larvae and especially larvae extract on fibroblast cell growth as the most important active cell in the healing of cutaneous wounds. Careful examination of the comparative protein expression between the control sample and the effective protein treatment revealed. The expression of the identified genes was then evaluated.

Materials and Methods

Cell culture

In this study, 3T3 mouse fibroblast cells were used to evaluate the changes of cells treated with the larval extract (cells obtained from Sabzevar Medical University). 3T3 fibroblast cells are highly functional cells that have been studied in a wide range of fields. These cells were grown in high glucose DMEM medium containing 10% fetal bovine serum and 1% penicillin/streptomycin antibiotic. 3T3 fibroblast cells were incubated at 37°C with 95% humidity and 5% CO₂.

Lucilia sericata larvae (prepared by Avan Khuzestan Company) were incubated in sterile conditions at 25°C for 72 hours. These larvae were fed raw beef that had reached room temperature before being consumed by the larvae. 160 larvae were washed with 110 ml of sterile distilled water and centrifuged at 2500 rpm at 4°C for 10 minutes. After centrifugation, the supernatant was separated and stored in -70°C freezer. After the larvae were completely frozen, they were removed from the freezer and completely homogenized with sterile glass rods. Then 120 ml of PBS was added to the larval homogenate and centrifuged at 5000 rpm for 4 minutes at 4°C and then filtered to obtain a bacteria-free extract in two steps, first 0.45 µm and then 0.22 µm filters were used and finally stored in a -70°C freezer.

Treatment of 3T3 fibroblast cells

One group of 3T3 fibroblast cells was considered as a control without any treatment and one group with a concentration of 12.5 µg/ml of larval extract treatment for 24 h according to our previous finding (Sanjari *et al.*, 2013).

2D electrophoresis

Protein extraction was performed by TCA/Acetone method. 100 grams of trichloroacetic acid was dissolved in 100 ml of distilled water and used with cold acetone to extract protein according to the original protocol. The Bradford method was used to determine the protein concentration and the BCA Protein Quantification Kit (Pars Tous) was used according to the kit instructions. The second-dimensional electrophoresis using PROTEAN II Xi under 32 mA for 30 min and 48 mA for 5 h, at 4°C until bromophenol blue reached 2 to 3 mm at the end of the gel. The gels were stained with silver nitrate and the protein spots were separated by molecular weight. The gel was scanned as soon as possible with Bio-Rad's GS800 densitometer using Quantity One

software and Image Master 2D Platinum (Powered by Melanie) software was used to quantitatively and qualitatively evaluate the spots. At the end of the two-dimensional gel protein stains (spots that had a significant difference between the control and the treated cells at a concentration of 12.5 µg/ml of larval extract) were identified by mass spectrometry (University of Singapore).

RNA extraction and real-time PCR

RNA was extracted from the Tripure solution of Bioneer Korea and then cDNA synthesized using Takara kit according to the protocol. The primers were designed by perlprimer software using the NCBI sequences for each gene and were synthesized by the Macrogen Corporation of South Korea (Table 1). Quantitative PCR was performed by the Rotor-Gen3000 instrument.

Table 1. Primers sequence and NCBI genome sequence code

Primer name	Sequences (5'→3')	NCBI Reference Sequence
GA3PD-F	5'-CATCACTGCCACCCAGAAGACTG-3'	NM_008084
GA3PD-R	5'-ATGCCAGTGAGCTTCCCGTTCAG-3'	NM_008084
Galectin-1-F	5'-GTAACACCAAGGAAGATGGGACC-3'	NM_008495
Galectin-1-R	5'-TCATGTCCGTCTGGCAGCTTGA-3'	NM_008495
β-actin-F	5'-CATTGCTGACAGGATGCAGAAGG-3'	NM_007393
β-actin-R	5'-TGCTGGAAGGTGGACAGTGAGG-3'	NM_007393
SOD-F	5'-GGTGAACCAGTTGTGTTGTCAGG-3'	NM_011434
SOD-R	5'-ATGAGGTCTGCACTGGTACAG-3'	NM_011434

Statistical Analysis

Expsy database was used to compare the results. GraphPad Prism 6 was used for statistical analysis and making statistical graphs. The results were compared to each other based on the one-way ANOVA test and TUKEY tests of Prism statistical software ($P < 0.05$).

Results

Culture of 3T3 fibroblast cells

Two-dimensional gel electrophoresis for 3T3 fibroblast cells as control and 3T3 fibroblast cells treated with larvae extract showed 12.5 µg/ml protein spots (Fig. 1). The number of recognizable spots by Image Master Software for different gels averaged over 100 protein spots. Examination of two-dimensional gel electrophoresis images showed that the fibroblast cells treated with larvae extract showed significant changes (increase or decrease in

intensity) compared to the control cells at 64 protein spots. The reason for these changes was the activity and effect of the larvae extract on the process of producing those proteins. From these spots, seven eye-catching spots were selected that were identified after isolation. The relative position of these spots is shown in figure 1 three protein spots were further investigated and identified. The presence of these spots in the control cell is much less pale but in the cells treated with larvae, the extract is easily detectable, indicating an increase in protein expression in the treated group. According to Fig. 1, spot number 1 is glyceraldehyde 3-phosphate dehydrogenase, spot number 4 is superoxide dismutase and spot number 5 is galectin-1 (Fig. 1). Expression of these proteins is increased in cells treated with larvae extract and their activity has a favorable effect on wound healing.

Galectin, SOD and GA3PD genes expression

Real-time PCR results showed good agreement with the results of 2D electrophoresis, confirming the increased expression of the target proteins (Fig. 2). The results of gene expression and the importance of gene expression mean that the larvae extract has an inducible effect on the increase of Galectin, SOD and GA3PD genes

expression and consequently due to the proteomics section, in general, has led to more expression of these proteins and optimal function in wound healing. In this study, the increased expression of genes confirms the results of the proteomics study. Gene expression significantly increased ($P < 0.05$) in comparison with the control group.

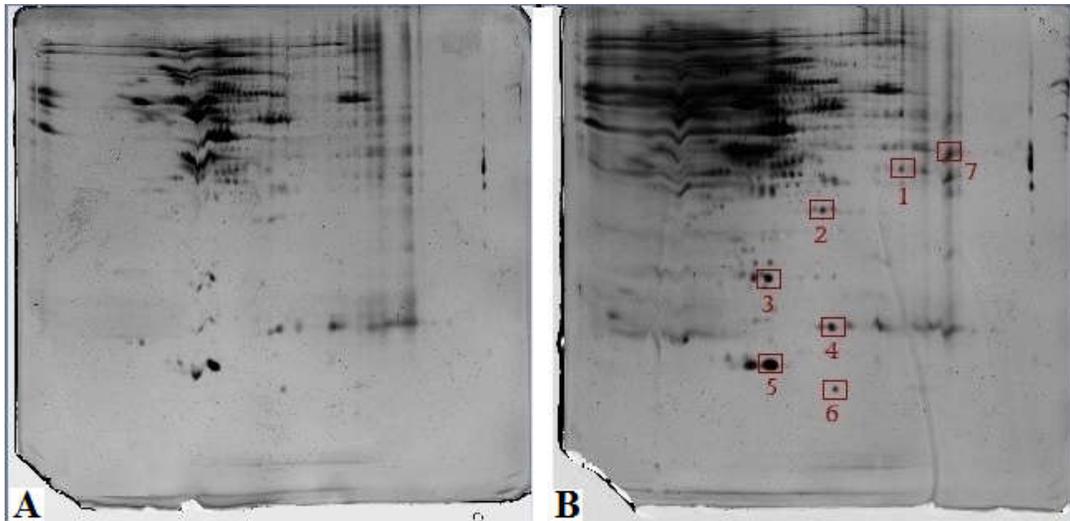


Fig. 1. Two-dimensional gel images: A) control group; B) larval extract-treated group; In the gel B, which indicates the expression of proteins in the treated group, spots were selected for sequencing, which showed the results of sequencing; Glyceraldehyde-1 phosphate dehydrogenase; Stain superoxide dismutase 4 and galectin-1 stain; all three proteins have an effective role in wound healing.

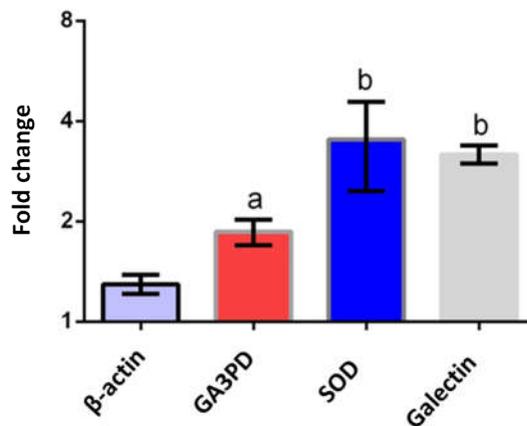


Fig. 2. Expression of target genes in the Real-time PCR: Y-axis is fold change of genes mentioned in the X-axis, GA3PD, SOD, and Galectin gene expression significantly increased ($p < 0.05$) in comparison with the control group.

Discussion

In different cellular pathways, there are essential control mechanisms that trigger the diverse activities in which glyceraldehyde-3-phosphate dehydrogenase participates and generates new functions (Sirover, 1999). Glyceraldehyde-3-phosphate dehydrogenase plays a key role in energy production. This enzyme is involved in mammals in membrane fusion, microtubule packaging, phosphotransferase activity, nuclear RNA export, DNA replication, and DNA repair. Observations indicate that there is a close relationship between new glyceraldehyde-3-phosphate dehydrogenase activity and cell pathology (Sirover, 2011). Glyceraldehyde 3-phosphate dehydrogenase regulates the activity of immune cells, thereby alleviating inflammation and wound healing (Foss *et al.*, 1998). In this study, increased production of this protein, which is related to the wound healing process, accelerates wound healing (Berry and Boulton 2000). Superoxide is a secondary byproduct of oxygen metabolism and, if increased in the cell, directly damages the cells, the result of this study confirms the increased expression of superoxide dismutase, which is one of the important enzymes in the cellular immune system. It is an antioxidant, increasing the expression of this enzyme in the present study indicates an increase in the antioxidant capacity of the fibroblast cells receiving the extract and this could be a very good strength for this extract.

Galectin 1 belongs to the family of beta-galactoside-binding proteins, less known than the other two proteins, but so far plays roles such as cell recognition, cellular attachment, and involvement in cell proliferation-related processes (Camby *et al.*, 2006). Also, Galectin 1 can induce muscle actin- α (α -SMA), fibronectin, and collagen (Col-1) expression in the wound area (Lim *et al.*, 2014). Galectin-1 itself is regulated by TGF (Lim *et al.*, 2014; Daroqui *et al.*, 2007), which has been demonstrated in various studies, our previous study showing that this protein increased expression during wound healing induced by larval extract, and the present study showed increased expression of galectin-1 itself. Further evidence is that the larvae extract has high

regenerative power with respect to the above proteins (Sanjari *et al.*, 2013). Studies have shown that different types of ROS-scavenging enzymes are usually produced at the site of the wound that plays an important role in wound healing and actually accelerates wound healing (Steiling *et al.*, 1999). Superoxide dismutase is also one of these enzymes that have been shown to increase gene expression as well as its increase in protein level in our study, and thus our previous studies on wound healing at *in-vivo* and *in vitro* levels may indicate the molecular function of larval extracts in wound healing (Sanjari *et al.*, 2013).

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Conclusion

The present study is the first study on the molecular level of the country regarding the differential expression of proteins in treated and control fibroblast cell samples. So far, larval therapy has been very limited, focusing on direct application to the wound, but this study seeks to determine the effect of the extract on the target tissue and indeed the fibroblast cells, which may be the most important cells in the wound healing process. The results indicated that at least three proteins had significantly increased expression in the treatment group, and the results showed that all three proteins were related to cell growth and proliferation processes and cellular communication. It may be due to the molecular cellular mechanism of the effect of the extract.

Conflict of interest

The authors have declared that no competing interests exist.

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