In Vitro Determination of Wound Healing Potential of Axonge

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Abstract: *Background*. Research on treatment alternatives that improve wound healing is an ever-evolving area in medicine, and a wound healing agent that carries minimal pain, discomfort, and scarring for patients with burn wounds, venous and decubitis ulcers, traumatic wounds, and many others is needed. The phases of wound healing include homeostasis, inflammation, migration, proliferation, and maturation. *Adeps suillus* (axonge) is known as a therapeutic agent for skin diseases and mainly consists of triglycerides. *Objective.* In the current study, the proliferation effect of axonge was determined on human normal epidermal keratinocyte (HaCaT) cells and human normal foreskin fibroblast cell line (BJ) cells. *Materials and Methods.* Experimental steps included preparation of HaCaT and BJ cell lines, axonge's stable tetrazolium salt-based proliferation assay, and evaluation of the wound healing effect of axonge on HaCaT and BJ cells. *Results.* Axonge concentrations of 3.12 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, and 50 µg/mL showed no cytotoxic effect on both HaCaT and BJ cells for 24, 48, and 72 hours. Considering the wound area of HaCaT cells, after 6 hours the wound healing effect of the axonge group reached almost 70% and then stopped. According to the results of the study on BJ cells, after 6 hours axonge wound closure was found to be 50% while the control group was only 10%. *Conclusion*. On the basis of this study, the authors determined that axonge might have potential for use in wound healing.

Key words: *Adeps suillus*, axonge, wound healing, proliferation, HaCaT cells

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Wound healing is a precise process of tissue growth and regeneration.[1] This process is composed of a series of overlapping stages in which a diversity of cellular mechanisms act together to regenerate the integrity of injured tissue and replacement of absent tissue.[2] Wound healing procedure comprises 5 phases of complex processes: homeostasis, inflammation, migration, proliferation, and maturation.[3] Research on treatment alternatives that improve wound healing is an ever-evolving area in medicine.[4] In numerous circumstances such as moderate to severe burns, venous and decubitus ulcers,

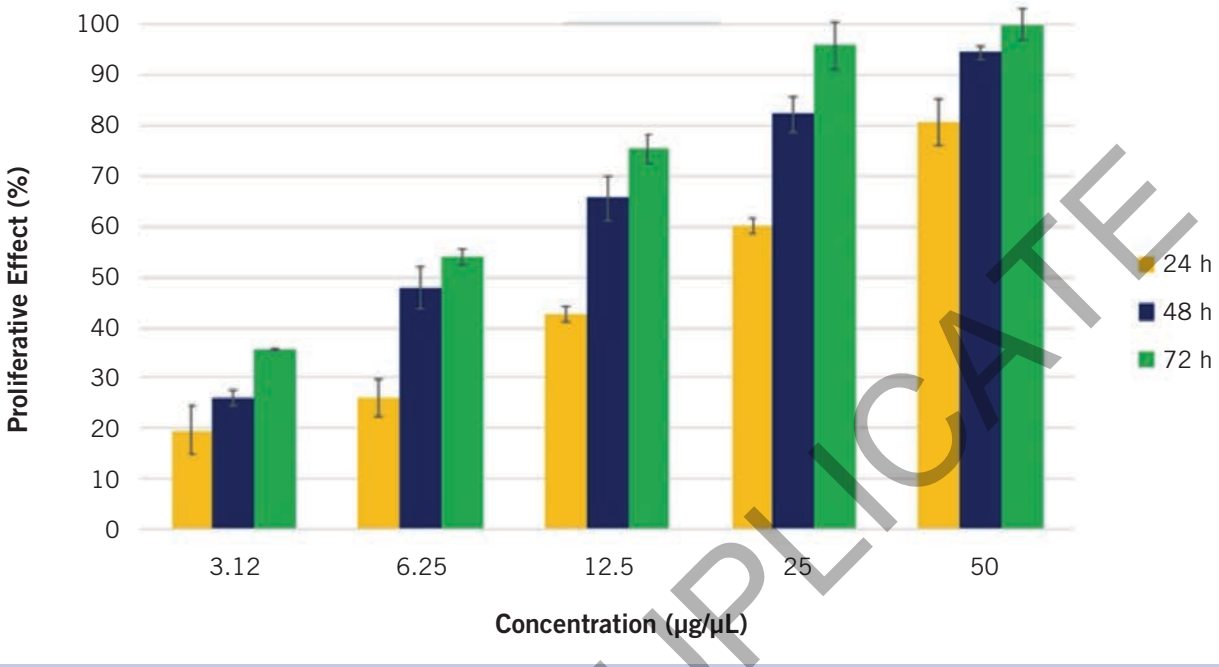

Figure 1. Cell proliferation of axonge on human normal epidermal keratinocyte cell line.

and trauma, an improved remedy is needed.[5] In addition, there is a necessity for a wound healing agent that causes minimal pain, discomfort, and scarring for patients.

Shiunko ointment is a traditional botanic formula used in clinical practice to treat multiple skin diseases for several hundred years in China.[6] *Adeps suillus* is 1 of 5 components (*Angelica sinensis*, *Lithospermi Radix*, *Oleum sesami*, *Cera Flava*, and *Adeps suillus*) of shiunko. *Adeps suillus* is also known as lard, *Axungia porci*, and axonge and is listed in the *Homeopathic Pharmacopoeia of the United States* (2013), an addendum to it, or its supplements; a homeopathic drug is any drug labeled as being homeopathic (alternative medicine).

The therapeutic effect of shiunko on granulation tissue formation includes speeding up wound healing, reepithelialization, and angiogenesis. In addition, Wu et al[7] showed its antibacterial and anti-inflammatory effects. The present study investigated the wound healing effect of *Adeps suillus* (axonge), which consists mainly of triglycerides. These triglycerides are composed of 3 fatty acids, and the distribution of fatty acids varies from oil to oil. In general, lard is similar to tallow in its composition.[8]

Petrovich[9] found the most effective dosage form for local wound care therapy is an ointment. It consists of a base (Constituens) and the drug evenly distributed into it. Petrolatum jelly (Vaselinum; Unilever, Englewood Cliffs, NJ), lanolin (Lanolinum), and purified pork fat are used for ointments as a base.[9] In the current study, the proliferation effect of axonge was determined on human normal epidermal keratinocyte (HaCaT) cells and human normal foreskin fibroblast cell line (BJ) cells.

Materials and Methods

Study design. Axonge was supplied from a pharmacy in Izmir, Turkey. It was packed by Novagenix (Ankara, Turkey) for the EGAS Medicine Device Co (Ankara, Turkey), with the certification number/date 35-51/01.08.2013 and lot number 72812. Ethanol was purchased from Merck (Kenilworth, NJ); Dulbecco's modified Eagle's medium (DMEM), L-glutamine, minimum essential amino acid, sodium pyruvate, fetal bovine serum, penicillin/streptomycin, trypan blue, phosphate buffer solution, and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Lonza (Basel, Switzerland). One miligram of axonge was freshly dissolved in 1 mL of ethanol in an ultrasonic bath prior to assays.

Preparation of human normal epidermal keratinocyte (HaCaT) cell lines and human normal foreskin fibroblast (BJ) cell lines. The HaCaT and BJ cell lines were obtained from CLS Cell Lines Service (Eppelheim, Germany). The HaCaT cells were grown in DMEM supplemented with 10%

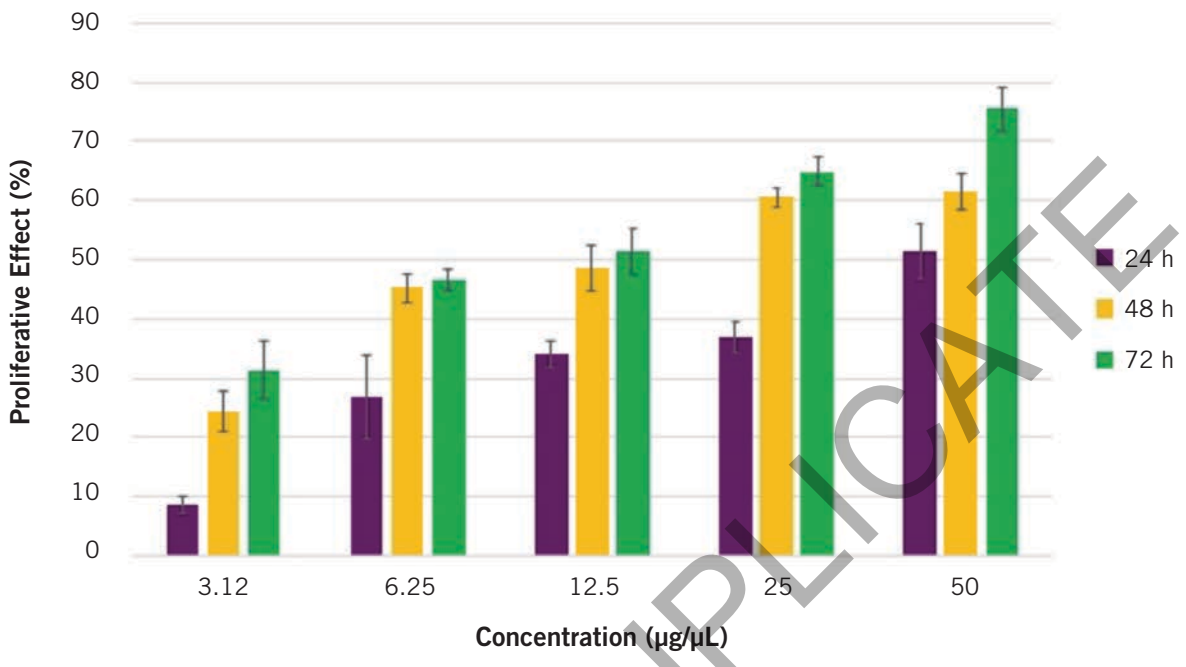

Figure 2. Cell proliferation of axonge on human normal foreskin fibroblast cell line.

fetal bovine serum, and BJ cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum at 37°C in a humidified incubator equilibrated with 5% carbon dioxide (CO_2). After cells reached the 80% mark on the flasks, they were suspended with trypsin-EDTA by enzymatic method.

Stable tetrazolium salt (WST-1)-based proliferation assay. Cell proliferation analysis was performed in 96-well plates using WST-1-based colorimetric assay according to the manufacturer's specifications (Roche Diagnostics, Mannheim, Germany). Each plate contained blanks, controls, and 5 dilution series of axonge with 3 replicates; 100 µL of cell suspensions (1 x 10^4 cells/well) were used in a 96-well plate. Stocks of samples (6.25–100 µg/mL) were prepared in culture medium. After 24, 48, and 72 hours of incubation at 37°C, 5% CO_2 was equilibrated in a humidified incubator and 10 µL of WST-1 reagent was added into the wells. In addition, 10 µL of DMEM with Earle's balanced salt solution was added into another well as the background control (blank). The absorbance was measured in a microplate reader (Varioskan Flash Multimode Reader; Thermo Fisher Scientific, Waltham, MA) at 450/620 nm.

Outcome parameters: wound healing effect of axonge on HaCaT and BJ cells. Concentration-dependent wound healing effects of axonge were analyzed according to scratch assay, which is previously described by Sanders et al.[10] The HaCaT and BJ cells (5 x 10^5 cells/well) were grown to 90% confluence in 24-well plates. Each well was scratched by a sterile pipette (200 µL) and marked below the plate surface by drawing vertical and horizontal lines for identification in order to take consistent photographs; the cells were grown in the 2 concentrations (0–50 µg/µL) of axonge. Treated cells (axonge group) and untreated cells (control group) were imaged between 0 to 96 hours by an inverted microscope (Leica Camera AG, Wetzlar, Germany), and wound healing potential of each sample was evaluated via Image J program (Version 1.45; National Institutes of Health, Bethesda, MD) with the following formula:

Wound Healing Potential =
(Healing Area/First Wound Area) x 100

Results

Cell proliferation of the axonge group on HaCaT and BJ cells is shown in Figures 1 and 2, respectively. Axonge concentrations of 3.12 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, and 50 µg/mL showed no cytotoxic effect on both HaCaT and BJ cells for 24, 48, and 72 hours (Figures 1, 2). Furthermore, each tested concentration of axonge

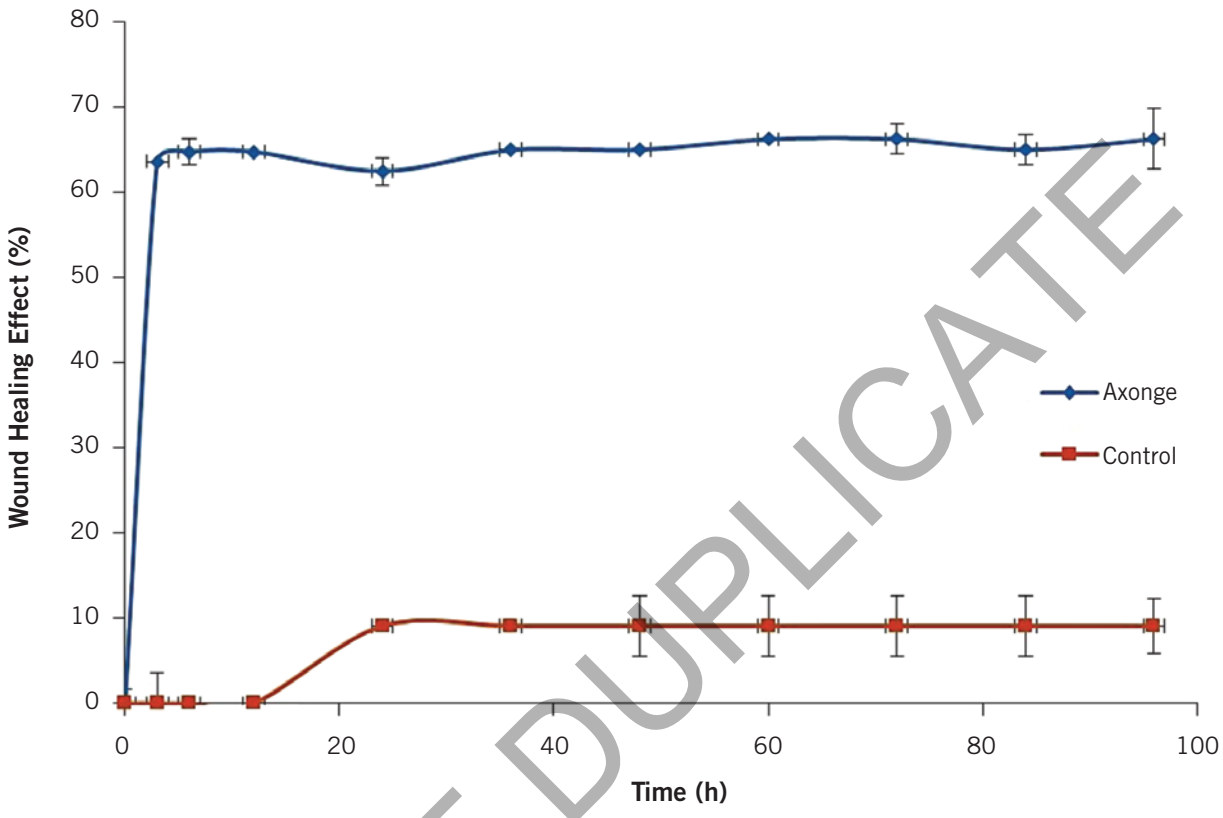

Figure 3. Time-dependent wound healing effect (%) of axonge on human normal epidermal keratinocyte cell line.

increased cell proliferation over those time periods. Cell proliferation percentages of 50 µg/mL axonge reached 100% and 70% for HaCaT and BJ cells, respectively. Time-dependent wound healing effects of axonge on HaCaT and BJ cells are shown in Figures 3 and 4. Considering the wound area of HaCaT cells, after 6 hours the wound healing effect of the axonge group reached almost 70% and then stopped; however, the control group did not show any effect on filling the defect area. Wound area of the control group started to heal after 12 hours, and by the end of 24 hours, the healing ratio stayed stable at about 10%. According to the results of the study on BJ cells, after 6 hours axonge wound closure was found to be 50% while the control group was only 10%. In addition, the wound healing effect of axonge after 24 hours reached 100%, and the control group was about 25%.

Discussion

In the present study, the investigators assessed the effects of axonge on the survival of HaCaT and BJ cells to better understand the effects of axonge on wound healing. The data showed axonge-based wound healing therapy is capable of accelerating wound healing and may have a potential for future applications.

Shiunko inhibits blood vessel permeability and delayed-type hypersensitivity, accelerates granulation tissue formation, and has antitumor and antibacterial activity.[11-14] In addition, when shiunko ointment is applied to a wound, it accelerates wound healing and inhibits capillary vessel permeability in rats.[11,12]

In a vehicle-controlled study,[14] shiunko was effective when compared with petrolatum, but not when compared with 3.5% saltwater. Results from a study by Higaki et al[15] suggest shiunko may have an antibacterial effect on staphylococci and may be an effective treatment primarily for suppurative skin diseases (eg, infected callus, hemorrhoids, eczema, and dermatophytosis) and more recently for atopic dermatitis and acne vulgaris. Miyakawa et al[16] reported that shiunko ointment was effective in treating atopic dermatitis in infants, especially in those with low

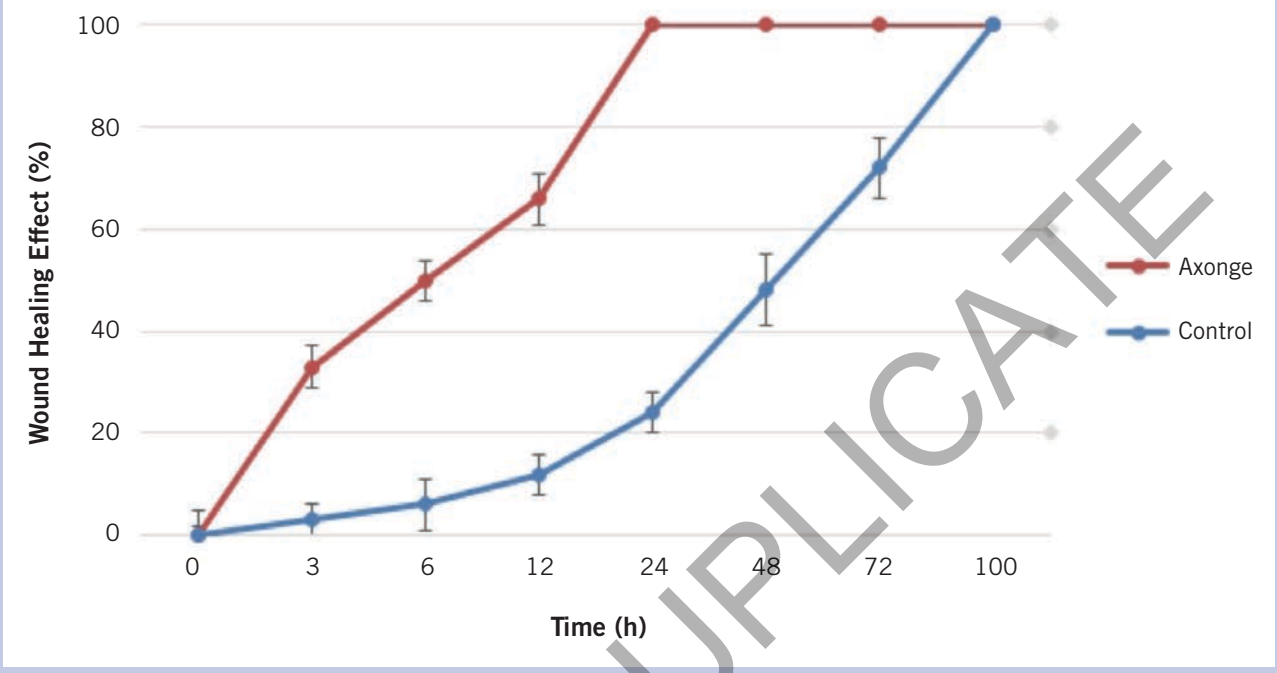

Figure 4. Time-dependent wound healing effect (%) of axonge on human normal foreskin fibroblast cell line.

levels of skin lipids in the epidermis and water in the stratum corneum. Ohkuma[17] showed that shiunko was an effective ointment for treating atopic dermatitis on the face and neck, but some unwanted side effects were noted, such as contact dermatitis, a foul odor, and fever. Further, Ohkuma[17] reported no adverse reactions on acne vulgaris. Mandelbaum et al[18] evaluated the in vivo process of tissue repair of experimentally induced cutaneous wounds in rats based on the kinetics of the regression of the ulcerated area over time. Magalhães et al[19] analyzed the effects of a combination of medium chain triglycerides, linoleic acid, soy lecithin, and vitamins A and E applied topically on this process in rats, and they considered the findings from earlier studies that demonstrated the efficacy of these agents in accelerating wound healing in human patients. In the present study, cell proliferation of axonge on HaCaT and BJ cells was determinate by WST-1-based cytotoxicity assay and revealed that each tested concentration of axonge increased cell proliferation over the study time period.

Woo-shin[20] examined the healing effect of Jawoongo plus *Rehmanniae radix* supplemented with axonge on rats and reported significant results after leukocyte, C-reactive protein, and cortisol measurements. The reduction of wound area was 87.2% after 15 days in this study.[20] In the present study, time-dependent wound healing effect (%) of axonge on HaCaT and BJ cells was investigated. Considering the wound area of HaCaT cells, after 6 hours, the wound healing effect of the axonge group reached nearly 70% and then stopped; however, the control group did not show any effect on filling the defect area. In addition, considering the wound area of BJ cells, after 24 hours the wound healing effect of axonge reached 100%, whereas this was about 25% in the control group. The results of both cell proliferation and wound healing studies were statistically compatible. However, proliferative and wound healing effects of axonge were more efficient for HaCaT cells than BJ cells.

In a study by Ho et al,[21] the authors evaluated "Jinchuang ointment," which contains 67% *Adeps suillus* on the HaCaT cell line, and showed cell proliferation in an in vitro cell line. In the same study, 3 cases with diabetic wounds and peripheral vascular disease demonstrated significant healing after the application of this ointment.

Limitations

This is the first study made on this topic, which has a lack of available data to support the findings. Moreover,

this is an in vitro study made with only 1 type of wound model. The number of samples is limited to the range of tested concentrations.

Conclusion

In conclusion, in vitro effects of axonge on HaCaT and BJ cell lines rationalized the remedial effect in wound healing. On the basis of this study, axonge may have potential for use in wound healing.

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