Original Article

The Effects of *Lucilia sericata* Larvae and *Eisenia fetida* Earthworm Extracts Either Alone or in Combination on Healing Third-Degree Burns in Male Mice

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Abstract

Background: Burn as the most common injury disrupts the protective function of the skin and induces complications in patients. Therefore, the treatment of these patients presents a significant clinical challenge. This study evaluated the effects of *Lucilia sericata* (*L. sericata*) larvae and *Eisenia fetida* (*E. fetida*) earthworm extracts, alone or in combination, on the healing of third-degree burns in male mice.

Materials and Methods: A third-degree burn model was induced on the skin of the interscapular region. Then, the extracts of larvae and earthworms were topically applied separately or simultaneously every other day for a 21-day period. To evaluate the process of wound healing, macroscopic parameters were monitored and examined during the study period. Finally, the animals were sacrificed, and skin sampling was performed for histological investigations.

Results: The results of the study showed that both extracts of larvae and earthworm accelerated the wound-healing process ($P < 0.01$). The group receiving extract of earthworm had better wound healing than the groups receiving Vaseline and silver sulfadiazine, and histological evidences confirmed these observations. However, the use of two extracts simultaneously did not affect the wound-healing process.

Conclusion: The results of this study demonstrated that the extracts of *L. sericata* larvae and *E. fetida* earthworm, especially *E. fetida*, include effective compounds that can significantly enhance the rate of burn wound healing. However, more studies are needed to identify and purify the effective compounds of these extracts involved in the process of wound healing.

Keywords: Burns, *Eisenia fetida*, extraction, *Lucilia sericata*, mice, wound healing

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Introduction

Burns are the fourth most common type of trauma worldwide.[1] Burn-related injuries are considered one of the most important causes of death among incidents endangering human health and life.[2] According to the World Health Organization (WHO) reports, there are 180,000 estimated deaths due to burns every year.[3] Burns are classified into four degrees, depending on the deep and intensity of penetrating the skin.[4-8] Third-degree burns as full-thickness burns are able to cause extensive damage to the skin and underlying structures, resulting in significant tissue inflammation, edema, and hypertrophic scarring.[9]

Various therapeutic interventions such as skin grafting, cell culture, and tissue engineering are employed in the treatment of burn wounds.[10-12] However, modern therapies are mostly accessible in developed countries due to their high cost.[13] In low-income countries with inadequate health care and low per

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capita health expenditures, there is a higher risk of delayed wound healing, which can be accompanied by complications such as infection, pain, disability, and finally death.[14] Consequently, cost-effective therapy methods are needed to progress burn wound healing and reduce overall costs. Larval therapy is a traditional alternative method for wound debridement.[15] Larval therapy, also known as maggot therapy, is a noninvasive method that has been proven effective in healing necrotic and chronic wounds,[16] including diabetic foot ulcers,[17] burns,[18] and bedsores.[19] In this therapy, *Lucilia* sericata (*L*. sericata) fly larvae, known as the green bottle fly, are placed on the wound to clean it and promote healing.[20] Studies have shown that larval secretions contain bioactive compounds that stimulate angiogenesis, boost immune response, and accelerate tissue repair.[21] Moreover, antibacterial, antifungal,[22] and anti-inflammatory effects have also been observed in these secretions that are important in the regeneration of damaged tissue.[21]

Eisenia fetida (*E. fetida*), known as the red earthworm, has been suggested as another non-medicinal method that may be effective in wound healing.[23] In ancient cultures, especially in traditional Chinese medicine, physicians would extract and utilize biologically active compounds from earthworms for medicinal purposes.[24] These compounds were used to treat various diseases such as burns, arthritis, itching, and inflammation.[25] The literature has documented the presence of bioactive compounds in *E. fetida* with antimicrobial,[26] anti-inflammatory,[27] and antioxidant effects,[28] making it a suitable candidate for burn wound healing.

Considering the limitations associated with the use of live larvae and the limited number of studies on the efficacy of larval and earthworm extracts, the objective of this study was to evaluate the effects of larval and earthworm extracts, either alone or in combination, on wound healing in burn injuries in male mice.

Materials and Methods

Animals

Twenty-five adult male BALB/C mice weighing 25 ± 7 g were used in this study. The animals were procured from the Isfahan University of Medical Sciences and housed in an animal room with standard conditions, including a temperature of 22 ± 2°C, humidity of 60–65%, and 12/12-hour light or dark cycle. They were provided with standard food and access to tap water *ad libitum*. The mice were acclimated to the experimental environment 1 week before the experiment. The procedures of study were approved by the Research and Ethics Committee of Isfahan University of Medical Sciences, Isfahan, Iran (IR. MUI.AEC.1402.031). Also, experimental procedures were performed in accordance with the health guidelines for the care and use of laboratory animals (National Institutes of Health Publication No. 80-23, revised in 1996).

Drugs

The drugs used in the research included ketamine (Trittau Co., Germany), xylazine (Interchem Co., Holland), sodium chloride 0.9% (Iranian Parenteral and Pharmaceutical Co., Tehran, Iran), silver sulfadiazine 1% cream (Sobhan Darou Co., Iran), Vaseline (Kanz Co., Iran), and morphine (morphine hydrochloride, TEMAD Tehran, Iran).

Extraction procedure

Sterile larvae of *L. sericata* were obtained from Zist Eltiam Sepanta, Isfahan, Iran. The larvae were incubated under sterile conditions and fed at a temperature of 25°C for 72 hours. After that, second- and third-instar larvae were separated. Then, 15 g of larvae weighed reached the volume of 100 mL in sterile distilled water and centrifuged for 10 minutes at 4°C and 2500 g. After removing the supernatant, the larvae were stored at -80°C. The frozen larvae were homogenized with a sterile glass rod and reached a volume of 100 mL by phosphate buffer solution (PBS) (0.02 M, pH 7.2). After mixing, the mixture was centrifuged at 5000 g for 10 minutes at 4°C. Finally, the supernatant with the concentration of 15% was removed.[29]

Earthworms were obtained from the breeding center of Pardis Kesht, Isfahan, Iran. The earthworms were placed in sterile distilled water for 24 hours. This step was performed to remove any mud that was stuck to their bodies and digestive systems. On the next day, the earthworms were washed several times to ensure they were clean. The washed earthworms were then frozen in a -80°C freezer. Once frozen, 15 g of the earthworms was homogenized using a glass rod. The volume of the homogenized earthworms was increased to 100 mL using a 0.02 M PBS with a pH of 7.2. The obtained mixture was then centrifuged at 5000 g for 10 minutes at 4°C. After centrifugation, the supernatant with a concentration of 15% was collected.

Finally, both extracts of *L. sericata* larvae and earthworms were transferred to a class II biological hood. To remove larger particles such as bacteria and viruses, the extracts were passed through filters with pore sizes of 0.45 μm and 0.22 μm (Merck, Millipore). This filtration process ensured the removal of any unwanted particles. Then, the extracts were stored at a temperature of -80°C for preservation. It is notable that both extracts were cultured on a blood agar medium to ensure a sterile environment before the experiment.

Burn wound model

Mice were anesthetized by intraperitoneal (IP) injections of ketamine (80 mg/kg) and xylazine (5 mg/kg). The dorsal surface area of the animals was shaved and disinfected with alcohol and cotton dipped in betadine solution. Then, a third-degree burn was induced by a stainless-steel rod with a diameter of 1 centimeter heated in water at 95°C for 5 minutes. The rode was placed on the shaved area for 10 S with equal pressure on all animals. To prevent dehydration, mice received saline solution (0.5 mL/kg, IP). Finally, the mice were individually kept in polycarbonate cages until the end of the experiment. To alleviate pain, morphine (10 mg/kg/day, IP) was administered before the burn and continued for 3 days thereafter.

Experimental design

After inducing the burn model, mice were randomly divided into five groups (n = 5) as follows: Vaseline group (negative control), 1% silver sulfadiazine group (positive control), group receiving larval extract, group receiving earthworm extract, and group receiving the combination of both larval and earthworm extracts (worm + larvae). All groups received topical treatment immediately after the induction of burn and continued every 48 hours for a 21-day duration. In the worm + larvae group, the use of *E. fetida* earthworm extract preceded. After 5 minutes (completing topical absorption), *L. sericata* larval extract was used. To evaluate wound healing, the digital photographs of wound area were taken on days 0, 3, 7, 14, and 21 after the induction of burn [Figure 1]. These photographs were then examined using image analysis software (ImageJ, NIH, MD, USA). The percentage of wound healing was calculated using the following equation:

wound area (%) = $A/A_i \times 100$

where A_i indicates the initial wound zone and A indicates the wound zone on days 3, 7, 14, and 21.

For histological assessment, the animals were anesthetized with ketamine (80 mg/kg) and xylazine (5 mg/kg). Then, the entire wounded area, along with a 1 mm border of surrounding tissue, was removed. After ending the experiment, all animals were euthanized using an overdose of ketamine and xylazine.

Histological evaluation

For histopathological investigations, the removed skin tissues were fixed in 10% formalin for 1 week. After embedding in paraffin, the tissue sections with a thickness of 5 microns were processed and stained with hematoxylin and eosin. The slices were observed and scored by a dermatologist in a blind manner using a light microscope, according to Table 1.

Statistical analysis

The data were statistically analyzed using Statistical Package for the Social Sciences (SPSS) version 23 software. To compare the within-group variables (changes on different days, day effect, and the trend of changes on different days based on groups, and day and group effect) and the between-group variable (group effect), a two-way analysis of variance (ANOVA), followed by a *post hoc* least significant difference (LSD) test, was performed. For the analysis of histological parameters, nonparametric analysis of Kruskal-Wallis or Mann-Whitney was performed. The data were presented as mean ± standard error of the mean (SEM), and P values < 0.05 were considered statistically significant.

Results

Figure 2 includes the comparisons of within-subject effects (day and day × group) and between-subject effects. The

Table 1: Histopathological evaluation by H and E staining

		0	1	2	3
Epidermis	Dermal-epidermal separation	Diffuse	Focal	None	–
	Hypergranulosis	None	Focal	Diffuse	–
	Crust formation	Present	Absent	–	–
Dermis	Edema	Severe	Moderate	Mild	None
	Fibroblast maturation	None	Mild	Moderate	Full
	Collagen amount	Abundant	Moderate	Scant	Normal
	PMN amount	Abundant	Moderate	Scant	None
	Depth of inflammation	Deep myonecrosis	Superficial myonecrosis	Lower dermis	Upper dermis

Pathology scoring system derived from a modified scoring system presented by Abramov *et al*.[30] considered and scored three and five criteria for the epidermis and dermis, respectively. The sum of the eight scores was considered to evaluate wound healing in each group

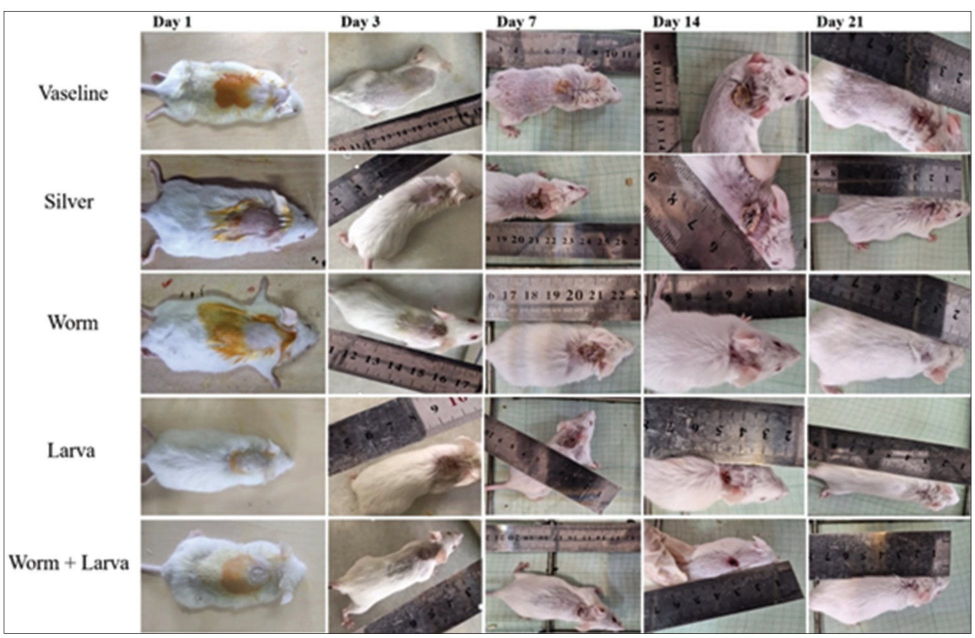

Figure 1: Macroscopic analysis of wound healing on different days. Digital photographs of mice showing various stages of wound healing on days 0, 3, 7, 14, and 21

findings demonstrated an improving trend in the percentage of wound area over time on days 1, 3, 7, 14, and 21 (day effect $F (4,72) = 227.65$, $P < 0.001$), which was different during the experiment period between the various groups (day × group effect $F (16,72) = 4.33$, $P < 0.001$). Also, there were significant differences in the percentage of wound area among the groups ($P < 0.01$). The comparison of experimental groups showed that the worm (0.61 ± 0.09, $P < 0.01$) and larvae (0.66 ± 0.1, $P < 0.01$) groups had significantly lower wound area percentages than the Vaseline group (0.79 ± 0.08). However, other groups showed no significant differences compared with the Vaseline group. In interest, the percentage of wound area showed a significant change in the worm group when compared with the silver group (0.77 ± 0.1, $P < 0.05$). Also, the worm and larvae groups had a significantly greater reduction in the percentage of wound area compared with the worm + larvae group (0.8 ± 0.1, $P < 0.001$ and $P < 0.01$, respectively). However, no significant difference was found in the percentage of wound area between the larvae and worm groups [Figure 2].

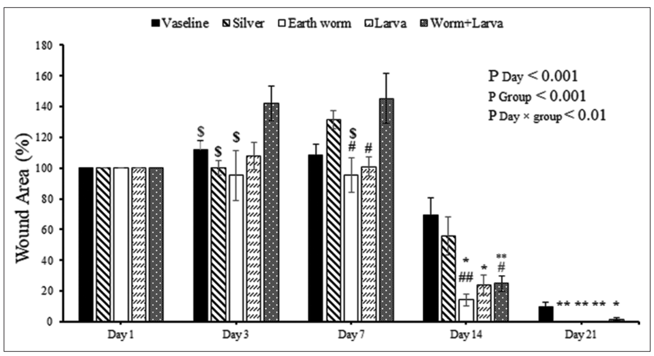

Figure 2: Comparison of the percentage of wound area in the experimental groups. The data were presented as mean ± SEM. $^{\$}P < 0.05$ and $^{\#}P < 0.05$ indicate significant differences from the worm + larvae and silver groups, respectively. * $P < 0.05$ and ** $P < 0.01$ indicate significant differences from the Vaseline group

The results of histological investigations in the experimental groups showed that the epidermis was uniformly formed in the group treated with earthworm extract. Although the maturation of fibroblasts and collagen deposition was visible in the dermis of all experimental groups on the 21st day of the treatment period, it was significant only in the group treated with earthworm extract. Also, the histological examination of the wound showed a significant decrease in inflammatory cells, neutrophils, and edema in the treated groups. This decrease was particularly significant in the group treated with earthworm extract ($P < 0.05$) [Figures 3 and 4].

Discussion

The study aimed to evaluate the effect of earthworm *E. fetida* and larvae *L. sericata* extracts either alone or together on burn-induced wound healing in male mice. In this study, a third-degree burn wound with a diameter of 1 cm confirmed by a histologist was created in all mice. Burn is a type of universal trauma model associated with severe pain, distress, and a reduction in quality of life, which influences personal and psychological aspects.[31] Therefore, it is important to find effective treatments to accelerate the healing process of burn wounds. Today, it is common to use natural products originating from plants or animals to improve various disorders, injuries, and traumas, such as burns. The extracts of larvae and earthworms are one of the natural products used in traditional medicine. In the current study, the animals were locally treated with extracts of earthworm, larvae, and combination of extracts for 21 days. The results demonstrated that earthworm extract significantly improved burn wound in comparison with groups treated with Vaseline and silver. The literature has documented that earthworm extract has antimicrobial and antibacterial properties.[32] The antibacterial activity of E. *fetida* has been observed against various bacteria, including *Salmonella typhosa*, *Staphylococcus aureus*, and

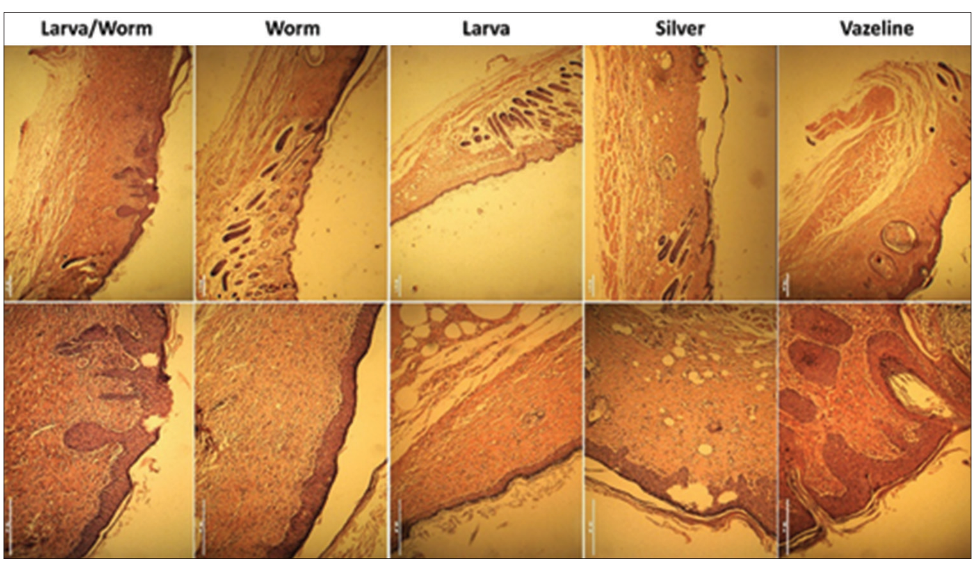

Figure 3: Skin histological images of wound regeneration. Representative images with hematoxylin and eosin (H and E) staining at day 21 post-wounding. Scale bar (10×), higher magnification scale bar (40×)

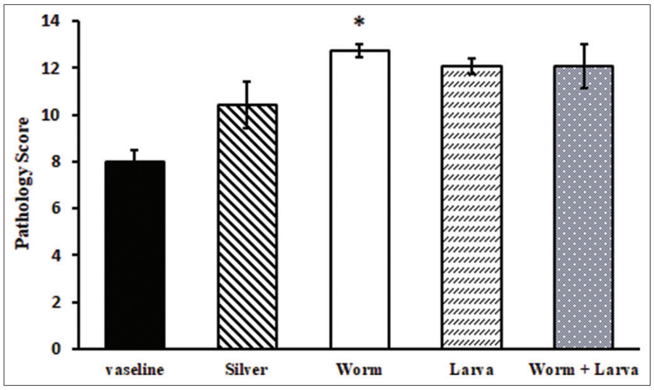

Figure 4: Histological assessment of regeneration of epidermis and dermis layers in the experimental groups. A higher score indicates more effective wound healing. Data were reported as mean ± SEM. The symbol * indicates a significant difference in comparison with the Vaseline group as the negative control group ($P < 0.05$)

Enterococcus faecalis. Evidence found two antibacterial peptides, F1 and F2, identified and isolated from earthworms. These peptides were able to exhibit antibacterial activity against both gram-positive and gram-negative bacteria, as well as fungi.[33] Therefore, it seems that earthworm extract has exhibited an improving effect by antibacterial activity in this study. Also, earthworm extract has antioxidant properties, which are important for wound healing.[34] The coelomic fluid of *E. fetida* contains a significant amount of phenol, which possesses antioxidant effects and can be utilized as a natural antioxidant for the treatment of diseases associated with inflammation and oxidative stress.[27] The effect of *E. fetida* extract as an antioxidant was investigated in cultured human fibroblasts and epithelial cells.[28] However, the present study did not explore the antioxidant effects of earthworm on burn injury. With regard to histological investigation, earthworm extract could regenerate the wound area and enhance the process of wound healing. One study has reported that earthworm extract increases the expression of epidermal and fibroblast growth factors, enhances collagen synthesis, and strengthens capillaries.[24] Also, it strengthens the immune system by increasing the production of white blood cells and platelets, which accelerates the removal of necrotic tissue.[35] In this way, it seems that this extract can accelerate wound healing and promote skin regeneration.

The present findings showed the effective properties of larval extract on wound healing. However, the use of live larvae may have psychologically adverse effects on patients.[36] Therefore, the use of larval extract as a treatment alternative may be more suitable for patients. Recent investigations have reported that larval extract has positive effects, including anti-inflammatory, antioxidant, collagen-producing, antibacterial, and antimicrobial properties, which contribute to speeding up the wound-healing process.[33,37] The substances found in this extract are able to reduce the number of harmful microorganisms and inhibit the growth of both gram-positive and gram-negative bacteria, including antibiotic-resistant strains such as *Staphylococcus aureus* and *Streptococcus pneumonia*.[32] Furthermore, the secretions of larvae contain proteolytic enzymes that can digest bacteria.[38] These antibacterial and antimicrobial effects contribute to reduce infection and promote wound healing. Another important factor in promoting wound healing is the regulation of inflammation and the concentration of growth factors. It has been shown that larval secretions contain substances that stimulate angiogenesis, growth factors, migration of fibroblasts to wound tissue, and collagen formation in wound tissue.[39] The presence of the P6 protein in the larval extract has been reported to facilitate wound healing by reducing the release of cytokines through the signaling pathways responsible for regulating angiogenesis and vascular permeability.[39] In a study, larval extract has been found to increase the levels of tumor necrosis factor-alpha (TNF-α), which is one of the essential factors in inflammation and indirectly improves inflammation.[40] Larval extract also increases the activity of antioxidant enzymes such as superoxide dismutase, which helps to reduce oxidative stress. Additionally, the extract can decrease the levels of malondialdehyde, a marker of oxidative damage, which is involved in wound healing.[39] It is recommended that further experiments be performed to exhibit the positive effects of larval extract in this burn model.

Finally, the present study showed that each extract alone was able to heal the wound. However, the co-administration of earthworm and larval extracts did not have a positive effect on wound healing. There are several possibilities: I. Each of the extracts exhibits effective properties at its specific pH. The co-administration of earthworm and larval extracts probably influences the pH of the environment and prevents or decreases each other's improving effects in the wound-healing process. II. It appears that the compounds in the two extracts can neutralize each other's effects or even prevent the desired treatment process by producing different products and metabolites. III. As a low concentration of oxygen-free radicals at the wound site can increase angiogenesis by inducing the expression of vascular endothelial growth factor in keratinocytes and macrophages, as well as stimulating collagen production,[41] it is important to have a low number of oxidative factors to promote wound healing. However, both extracts have antioxidant effects. Therefore, the cumulative effects of both extracts not only failed to ameliorate burn injuries, but the effects could also disturb the wound-healing process. It should increase the interval of co-administration of earthworm and larval extracts to prevent harmful interactions of both extracts.

Conclusion

This study concluded that two extracts of *L. sericata* larvae and *E. fetida* containing active substances can improve burn wounds and accelerate wound healing. In addition, this finding is also promising for repairing other types of tissue damage. However, further studies are needed to identify the active compounds of these extracts and understand the mechanisms involved in the treatment of various types of tissue damage.

Acknowledgment

This research has been scientifically and ethically approved by the Isfahan University of Medical Sciences.

Ethical statement

The Research Ethics Committee of the Isfahan University of Medical Sciences (IR.MUI.AEC.1402.031) approved all experiments.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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