Abstract. — Background and Objectives: This study was aimed at demonstrating the influence of *Eisenia foetida* earthworm extract (G-90) on wound healing in an animal model. Medicinal properties of earthworms have been long known, especially in Eastern countries.

Materials and Methods: Four groups of Wistar rats (36 in each group) were wounded under anaesthesia; the skin was surgically incised in a circular manner (the circular incision in reference measuring 2 cm in diameter) and afterwards daily treated for 24 days in a following manner: Group 0 – the control group deprived from any treatment whatsoever; Group 1 – treated with physiological saline solution; Group 2 – treated with Panthenol D and serving as a positive control, and Group 3 – treated with G-90 (10 ng/ml). The animals were sacrificed on days 3, 6, 9, 12, 18 and 24 post wounding and the diameter of wound was measured by virtue of photometric method. The excised wounds were routinely fixed and embedded into paraffin section and duly stained for histopathological analysis. The presence of microorganisms on the wounds was assessed as well.

Results: The best antibacterial wound shielding was achieved with G-90 treatment. Besides antibacterial shielding, the wounds treated with G-90 were also protected from inflammation. G-90 was shown to shorten the inflammatory, and accelerate the proliferative and the maturation phase, stimulating thereby the regeneration of an injured epidermis. Statistical analysis revealed G-90 (p=0.018) to be superior over other treatments.

Conclusions: Thus, *Eisenia foetida* earthworm extract (G90) might be considered as a new wound-healing agent suitable for use in both veterinary and human medicine practice.

Key Words:


Introduction

Many scientists and medical communities have been searching for way to improve wound care; it seems that they have tried everything in their power to promote wound-healing. In order to obtain the information on multifactor nature of the wound-healing process, one should inevitably use some kind of a model. However, the process may also be influenced by external factors. The most substantiating initial test to be employed with any factor affecting the wound-healing process, are animal experiments *in vivo*. One of the advantages of an animal model is that the wound-healing process is accelerated, so that it can be studied over days rather than over weeks, as necessary in human studies.

Skin wound healing is a complex process characterized by re-epithelization and restoration of the underlying connective tissue. A number of overlapping phases are involved. During this process, keratinocytes, endothelial cells, fibroblasts and inflammatory cells proliferate and/or migrate to the site of injury, interacting both with each other and with extracellular matrix. Cell migration and tissue remodeling which take place during the course of the wound-healing process require controlled degradation of extracellular matrix and activation or release of growth factors. Along the line of neovascularisation, matrix-generating cells move into the granulation tissue. These fibroblasts degrade the provisional matrix and respond to cytokine/growth factors by proliferating and synthesizing new extracellular matrix.

Within the frame of this study, the preparation obtained from tissue homogenate of the earthworm *Eisenia foetida*, named G-90, had been employed. The use of earthworms for medical purposes was documented at a very early date, even back in 1340 A.D. Traditional Indian healers operating in North America and doctors in East Asia have used earthworms in order to treat various diseases. An Asian study made a great contribution to better understanding of medical use of
earthworm preparations\(^9\). In recent times, biochemists have been searching for natural sources of active components capable of curing different diseases and healing wounds. Wound-healing agents commonly used insofar originate from plants rather than animal sources. Folk medicine makes use of several wound-treating plants, e.g. *Hypericum hookerianum*\(^10\) and *Hypericum patulum*\(^11\), *Carica papaya* L.\(^12\), *Matricaria recutita* L.\(^13\), *Aspilia africana*\(^14\), etc. Recently, the earthworm paste (*Lampito mauritii*, Kinberg) has been launched, exhibiting anti-inflammatory and antioxidative properties and influencing haematological parameters, all of the aforementioned being important for the wound-healing process\(^15\),\(^16\). It has also been reported that earthworm coelomic fluid contains molecules that exhibit cytolytic, agglutinating, proteolytic, haemolytic, mitogenic, antipyretic, and antibacterial properties\(^17\)-\(^19\).

Fifteen years of studying the properties of *Eisenia fetida* earthworm extract (G-90) have resulted in the body of knowledge which could contribute to the advancements in the wound-healing process. However, no attempts as to study the influence of earthworm extracts on wound-healing have been made insofar. We would like to fill that gap by showing that, during the course of skin wound healing, G-90 macro-molecules influence tissue regeneration and accelerate proliferation and synthesis of growth factors. Previous studies of G-90 impact have shown that it exhibits different properties, such as mitogenic\(^20\), fibrinolytic\(^21\), anticoagulative\(^22\), antioxidative\(^23\), antibacterial\(^24\) ones and stimulates transforming growth factor (TGF) and epidermal growth factor (EGF) synthesis\(^25\). All of these properties and action modalities may contribute to the acceleration of the wound-healing process. In this study, the influence of G-90 on wound-healing process was examined in comparison to that of Panthenol D, already well-established and commonly used wound-treating agent.

**Materials and Methods**

**Animals**

Adult Wister rats were obtained from the Institute for Medical Research and Occupational Health, Zagreb, Croatia. The procedure used in this study was in accordance with the provisions of the National Law on Care and Use of Laboratory Animals, and was approved by the Board of Ethics in charge of Animal Care & Use, operating under the wing of the Institute for Medical Research and Occupational Health. The animals were kept in steady-state micro-environmental conditions (22 ± 1°C, 50-70%-humidity), had received standard laboratory food and water *ad libitum*, and were exposed to alternating 12h-lasting light/darkness cycles. Two individual experiments were performed, each involving 72 animals divided into four groups of 18 (males: females ratio, 1:1); individual animal weight 200-300 g.

Each rat was introduced into anaesthesia using 1.4 ml/kg of an anaesthetic drug (1 ml Narketan + 0.75 ml Xylapan, Vetoquinol, Switzerland). Rat’s dorsal skin was shaved and cleaned with 70%-ethanol, and subsequently surgically incised in a circular manner (the circular incision in reference measuring 2 cm in diameter) up to the subcutaneous adipose tissue level. The animals were treated on an everyday basis in the following manner: Group 0, i.e. the control group, was deprived from any treatment whatsoever; Group 1 was treated with physiological saline solution (which has the potential of dissolving G-90); Group 2 was treated with Panthenol D (Jadran Galen Laboratory, Rijeka, Croatia) and posed as the positive control; Group 3 was treated with G-90 (10 ng/ml). On days 3, 6, 9, 12, 18 and 24 postwounding, 6 animals from each group were sacrificed by virtue of intraperitoneal administration of T 61 (Intervent, the Netherlands). The wound area was measured photometrically using the additional check points (morphometric evaluation). The entire dorsal skin encompassing healing wounds was excised. The samples were submerged into formalin for histological and histochemical analyses. The experiment was repeated twice, and was preceded by a pilot.

**Preparation of G-90**

Glycolipoprotein mixture (G-90) was obtained from tissue homogenate of *Eisenia fetida* according to the procedure described by Hrzenjak et al\(^26\). Water-soluble powder containing 40 µg of proteins/mg powder, was dissolved in sterile saline (0.9% NaCl) and diluted to the final concentration of 10 ng/ml. This solution was used for wound treatment.

**Microbiological Wound Screening**

Prior to the sacrifice, wound samples intended for microbiological analysis were taken using a sterile cotton stick. Border and middle wound por-
tions were wiped and the samples were then evenly spread across the agar plates. Bacterial growth was estimated after a 24 h-lag period at 37°C. Bacterial colonies were counted in numbers, while the results were expressed as means obtained in 3 parallel samples within two independent experiments.

**Histological Analyses**

Tissue samples taken from skin wounds were cut through the middle of the wound, preserving thereby the skin on the edges, and fixed in buffered formalin. Wound samples were embedded into paraffin “sideways-on”; tissue sections (0.5 µm thick) (N = 432) were stained with haematoxylin, eosin, Mallory stain and van Gieson stain as due for the target tissue aimed to be identified. Tissue samples were analyzed using an Olympus BH2 light microscope (Lawrence, Kansas, USA). The edge of the wound was defined as the area hosting cellular connective dermal tissue, while the wound base was defined as the area hosting mature adipose tissue. Morphometric evaluation of the re-epithelization process was performed using an Olympus BH 2 microscope equipped with digital microscope camera Olympus DP10, while the results were processed using Olympus DP-Soft program. The percentage of re-epithelization was calculated for each and every wound according to the following equation:

\[
\text{Length of the wound covered} \\times 100
\text{with a new epithelial lining}\]

\[
\text{Length of the initial wound}
\]

Pathohistological analysis was carried out in a semi-quantitative fashion, and each wound was scored relative of its healing progress on a 0-3 scale. The dynamics of the healing process was estimated semi-quantitatively, taking into account:

1. The type (mature/immature) and the amount of granulating tissue (score 0-3);
2. The amount of the connective tissue – fibrosis (score 0-3);
3. The amount of the mature collagen – Co (score 0-3).

Granulating tissue was clustered and defined in the following manner:

- immature granulating tissue (IM): soft granulating tissue (macrophages, fibroblasts) accompanied by newly-formed blood vessels;
- mature granulating tissue (M): fibroblasts and sparse extracellular matrix proteins, with blood vessels running perpendicularly;
- Granulating tissue grading: 0 – Absence of granulating tissue.
  - Granulating tissue filling only a small part of the wound bed.
  - A thin layer of granulating tissue spreading wound bed-wide.
  - A thick layer of granulating tissue spreading wound bed-wide.
- Fibrosis (F): dominated by extracellular matrix proteins containing fewer blood vessels and fibroblasts. Its presence was determined semi-quantitatively and scored on a 0-3 scale, as with granulating tissue.
- Mature collagen (Co): bundles of collagen stained red in van Gieson fashion (determined semi-quantitatively and also scored on a 0-3 scale).

An inflammatory infiltrate was defined as the presence or absence of the specific type of inflammatory response, in terms of the following:

- a non-specific inflammation: an inflammation not associated with the wound-healing process and characterized by the accumulation of polymorphonuclear leukocytes;
- a specific inflammation: an inflammation associated with the wound-healing process and characterised by the presence of neutrophils and sometimes eosinophils “tangled” within the granulating tissue).

The reagents used for histological staining and all other chemicals employed were p.a. grade, manufactured by Kemika, Zagreb, Croatia.

**Statistical Analysis**

Statistical significance between the experimental and the control values was analyzed using Kruskal-Wallis ANOVA test. P-values of <0.05 were considered statistically significant.

**Results**

**Wound Closure**

The percentage of wound closure was calculated as the ratio of the magnitude of the wound surface area over time- since-wounding. Summarized results of the two independent experiments
are shown in Figure 1. On day 12, the percentage of the closure of wounds inflicted to the control rats, the rats treated with physiological saline solution, and those treated with Panthenol D, was estimated at approximately 50%, 60% and 40%, respectively. At the same time, the percentage of closure attained in wounds treated with G-90 was approximately 70%. On the average, wounds inflicted to G-90-treated rats closed faster as compared to other animal groups under observation (re-epithelization occurred 1.2 to 1.75 times faster than in other animal groups).

**Microbiological Wound Analyses**

The results of microbiological analyses are shown in Figure 2. The samples were taken on the sacrifice day and analyzed after 24 h. Bacterial colonies’ count varied across the wound-healing period. The maximum was reached at days 6 and 9 post wounding. The best anti-bacterial wound protection was achieved with G-90 treatment. Besides anti-bacterial protection, the wounds treated with G-90 were also protected from inflammation. All wounds were almost dry and healed after 18 days.

**Histological Analysis**

Histological analysis of 432 wound samples (144 animals × 3 samples taken from each of them) revealed differences between the analyzed animal groups, apparent on day 6, 9 and 12 post wounding, in terms of both the amount and the type of newly-formed granulating tissue (immature/mature), as well as the formation of mature connective tissue, i.e. fibrosis). Figure 3 displays

![Figure 1](image1.png)

**Figure 1.** Time-course of wound closure. Wound closure rate was calculated as the percentage of surface area magnitude over time-since-wounding. The results were statistically analyzed by virtue of Kruskal-Wallis ANOVA method and expressed as median values (*p* < 0.05).

![Figure 2](image2.png)

**Figure 2.** Microbiological analysis of the wounds inflicted on the rat’s skin and treated in the following manner: none (the control group), physiological saline solution, G-90, and Panthenol D. The samples were taken on days 3, 6, 9, 12 and 18 post wounding. The results are given as mean values pertinent to three parallel samples. The Figure displays one representative of each three-sample cluster.

a twelve day-old wound filled with granulating tissue and partially re-epithelised. The observed differences had manifested themselves in form of an enhanced maturation and formation of granulating tissue. The semi-quantitative scores allocated based on the presence of the mature granulating tissue, were consistently higher in G-90 treated rats (days 6, 9, 12) in comparison to the control and the rat groups treated with physiological saline and Panthenol D (Figure 4). Furthermore, the formation of mature granulating tissue, observed among G-90 treated rats, reached its peak on day 12, while in Panthenol D-treated rats this occurred two weeks later. In G-90-treated rats the presence of fibrosis (mature connective tissue) was noticed as soon as on day 9, i.e. at the earliest time-point as compared to other differently treated animal groups (Figure 4).

Statistical significance calculated by virtue of Kruskal-Wallis test (*p* < 0.05), pertinent to the formation of immature granulating tissue, was attained on day 6, while that pertinent to the mature tissue was attained on days 6 and 9, and that pertinent to the fibrotic tissue and collagen on day 24.

The difference in the re-epithelisation rate established among the observed groups was most pronounced on day 12. It reflected itself in a significantly larger percentage of wound area covered by newly-formed epithelial lining, encountered in G-90-treated rats in comparison to the remaining three groups (Figure 5). Re-epithelization of wounds and enhanced dynamics of the
healing process were statistically significantly more pronounced in the rats treated with G-90 \((p = 0.0188)\) then with other treatments.

**Discussion**

G-90 is a glycolipoprotein mixture prepared from a tissue of the earthworm *Eisenia fetida*. Earlier investigations of G-90 glycolipoprotein mixture have shown that this mixture is capable of “undertaking” different activities, such as mitogenic\(^{20}\), fibrinolytic\(^{21}\), anticoagulative\(^{22}\), antibacterial\(^{23}\), and anti-oxidative one\(^{24}\), as well as of stimulating the synthesis of TGF and EGF\(^{25}\), which could all contribute to the rapidness of wound-healing. Mitogen activity of G-90\(^{20}\) could be responsible for the proliferation of fibroblasts and epithelial cells, contributing in that manner to the celerity of the wound-healing process. Antibacterial\(^{23}\) and antioxidative\(^{24}\) impact of G-90 managed to prevent an inflammation onset. Also, the experiments with mice have shown that G-90 stimulates the synthesis of EGF and fibroblast growth factor (FGF)\(^{25}\), i.e. the synthesis of growth factors which contribute to the higher fibroblast and epithelial cell proliferation rates. The results of our study suggest that wound healing was positively impacted by G-90 in all of the process aspects. Re-epithelization of wounds treated with G-90, determined by virtue of the wound area measurements, showed that the time needed for wound closure was shortened (Figure 1). Histological analysis in terms of granulating, connective and collagen tissue measurements, had revealed the potential of G-90 treatment to increase the synthesis of the above-mentioned (Figures 3, 4, 5). Healing potential exhibited by G-90, together with its other biochemical and biomedical properties\(^{8,29}\), might be of therapeutic importance. Thus, G-90 could be very useful as an animal-sourced agent and pose as a good candidate for animal or human wound healing-targeted treatment. Scheme 1 demonstrates the potential role of G-90 in the wound-healing process. The earthworms are at disposal virtually

![Figure 3](image-url)
everywhere and the preparation of glycolipoprotein mixture (G-90) is very cheap and simple. However, many additional proofs should be presented prior to its use as a wound-healing agent.

Insofar, agents used for wound treatment have been mainly extracted from different medicinal plants, to the goal of which leaves, flowers or stems of the latter have been harvested. Knowledge about agents having a biomedical potential that allows for wound care, stemming from animal sources, is very sparse. Homogenates/pastes originating from earthworms have been reported to have some characteristics that could contribute to better wound healing. Mitogenic, antibacterial, haemostatic and anti-oxidative impacts have a major influence on wound healing and epithelization. The paste obtained from the earthworm Lampito mauritii, Kinberg, has exhibited a variety of properties, such as anti-inflammatory, antioxidative and hepatoprotective ones. In line with their properties, the use of animal extracts should be considered not only in wound, but in various human disease treatments. The use of a natural product derived from plants or animal sources, offers the possibility of exercising a new approach both in comparative and alternative medicine settings. The implementation of some of these compounds into the treatment of human and animal diseases might as well be set as the goal that both scientists and experts engaged into comparative and alternative medicine should strive to achieve. Scientific approach should give solid evidence supportable of use of these preparations already long-exploited in the folk medicine settings.

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**Figure 4.** Formation of granulating tissue area: Immature tissue, Mature tissue, Fibrotic tissue, Collagen tissue, evaluated on a 0–3 scale in the control (0), physiological saline-treated (1), Panthenol D-treated (2) and G-90-treated (3) rats (Details provided in the “Materials and Methods” section). The results were statistically analyzed using Kruskal-Wallis ANOVA method and are displayed as median values (*p < 0.05).

**Figure 5.** Re-epithelization (%) of wounds after the following treatment: 0 – none, 1 – with physiological saline solution, 2 – with Panthenol D, 3 – with G-90 (10 ng/ml). The results were statistically analyzed using Kruskal-Wallis ANOVA method and are displayed as median values (*p < 0.05).
Conclusions

Earthworms represent a very convenient source of natural bioactive molecules which could prove the selves useful in both human and veterinary medicine settings. Thus, glycolipoprotein mixture (G-90), with its versatile biological potentials, represents a good candidate for a wound-curing agent.

References


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