Bioelectrical Stimulation Potentially Reduces Bleeding Time and Accelerates Regeneration in *Lumbricus Terrestris*

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Background: Complications from bleeding and wounds are health problems that lead to serious morbidity and mortality, especially for those with chronic conditions. Bioelectrical stimulation may be a potential therapy to induce vasoconstriction and accelerate wound regeneration through neuromodulation. The objective of this experiment was to determine the effect of bioelectrical stimulation on bleeding and regeneration in *Lumbricus terrestris*.
Methods: The experimental group ($N = 15$) was stimulated using a transcutaneous electrical nerve stimulation device (3 min, intensity 2.20 Hz) in 100 ml of distilled water. The control group ($N = 15$) did not receive electrical stimulation. For bleeding time, earthworms were amputated at the eight segments of the head. The bleeding time and bleeding area were recorded. For regeneration, earthworms were amputated at the eight segments of the tail and injected with Visual Elastomer Implant dye to mark the amputation site. The length of regenerated segments was measured for six days.
Results: Bleeding time of the experimental group (1:15$\pm 6 \times 10^{-5}$ min) was significantly shorter than the control group (2:04$\pm 3 \times 10^{-4}$ min), P=0.009. Bleeding area was not significantly different between groups. Regeneration of the experimental group (3.73$\pm 1.72 mm) was significantly longer than the control group (1.19 \pm 0.27 mm), P = 0.001$.
Conclusions: Bleeding time was shorter and regeneration length was longer in earthworms exposed to bioelectrical stimulation. Bioelectrical stimulation may be a promising technology to accelerate the recovery of wounds.

Keywords: bioelectronic medicine, neuromodulation, wound healing, hemostasis, *Lumbricus terrestris*

Introduction

Wounds, such as punctured skin, cuts, scratches, and scrapes, are defined as injuries that break the skin or other body tissues[1]. Oftentimes, wounds lead to hemorrhage or infection. Complications from blood loss and wounds are health care problems that lead to significant morbidity and mortality, especially for those with chronic conditions such as diabetes and those undergoing surgeries. It is estimated that 60,000 Americans die each year from hemorrhages. Worldwide, an estimated 1.9 million people die each year from blood loss with 1.5 million of those deaths being caused by physical trauma[2]. In the United States, Medicare spent an estimated $32 billion on wounds in 2014[3]. In 2017-2018, the United Kingdom National Health Service spent an estimated 8.3 billion pounds on 3.8 million wound cases[4]. More effective approaches to control bleeding and accelerate recovery of wounds are needed to combat these significant health issues.

Bioelectronic medicine is a growing field of study that uses medical devices to generate electrical pulses, working with the body's natural mechanisms to cure diseases[5]. An example of this is bioelectrical stimulation, whereby electric signals stimulate the nerves and body to help treat medical conditions6. It has been suggested that bioelectrical stimulation may be beneficial for reducing bleeding time and accelerating wound healing through neuromodulation. By altering the endogenous electric fields surrounding wounds, bioelectrical stimulation may potentially reduce blood flow by inducing vasoconstriction within arteries and blood vessels and help the wound regeneration process[6]. A study on the effect of electrical stimulation on vasoconstriction in rats found that low voltage electrical stimulation caused the arteries to constrict by 6-8%. Meanwhile, high voltage stimulation caused the arteries to constrict by 11-18%[7]. Another study showed that vasoconstriction was induced by electrical stimulation in rats, which led to blood clot formation and decreased bleeding time[8].

Bioelectrical stimulation is also believed to help the wound regeneration process by directing electricity to the center point of the wound. It can also affect the endogenous electrical field around a wound, thus affecting the inflammation phase, blood flow, cell proliferation and migration, and scarring[6]. In a study of crawfish, it was found that electrical stimulation was able to bring immunocytes (microphages, lymphocytes, and neutrophils) towards the wound, thus speeding up the healing process[9]. According to a pilot study done by Olivera et al, bioelectrical stimulation on chronic wounds in humans for 30 minutes a day reduced wound surface and alleviated pain. Increasing the duration to an hour a day for three days improved blood flow[10]. These studies support the use of bioelectrical stimulation for the treatment of wound healing, however the effect of bioelectrical stimulation on stem cells and regeneration of tissue is understudied.

Investigating the potential of bioelectrical stimulation as a therapeutic intervention for wound healing may have significant impact on public health. The objective of the study was to examine the effect of bioelectrical stimulation on the bleeding time and regeneration process of the earthworm, *Lumbricus terrestris*. Earthworms are an ideal animal model to study the pathophysiology of wound healing since, like humans, they have a circulatory system that is made up of blood vessels with circulating red blood cells that contains hemoglobin[11,12]. Furthermore, earthworms have the universal ability to fully regenerate any lost segments, offering opportunities to study tissue and stem cell properties, which are advantages over previously studied animal models[13]. Earthworms are also a more cost effective and non-vertebrate alternative animal model. In this study, it was hypothesized that earthworms that received electrical stimulation prior to amputation would have shorter bleeding times and longer regeneration length compared to those that were not electrical stimulated.

Results

The bleeding time of the experimental group ($N = 15$) was compared to the control group ($N = 15$). The bleeding time of the experimental group (1:15$\pm 6\times 10^{-5}$ min) was significantly shorter than the control group (2:04$\pm 3\times 10^{-4}$ min), P value = 0.009 (Figure 1).

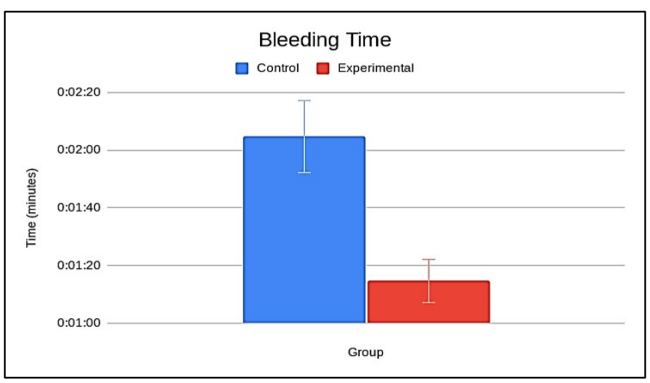

Fig. 1 Bleeding time (min) of the experimental group (3 groups, N=15) compared to the control group (3 groups, N=15), P=0.009. (Graph by authors, 2023)

The area of blood loss of the experimental group (398$\pm$197 mm^2, N=5) was not significantly different from the control group (723$\pm$421 mm^2, N=5), P value = 0.2 (Figure 2).

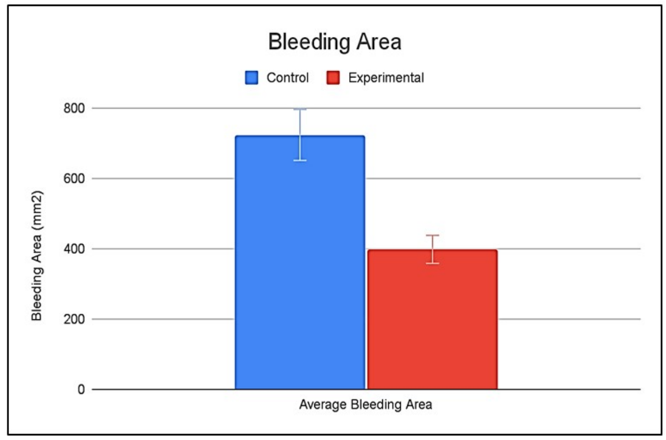

Fig. 2 Area of blood loss of the experimental group (1 group, N=5) was not significantly different from the control group (1 group, N=5), P= 0.2. (Graph by authors, 2023)

Regeneration length of the experimental group (3.73$\pm$1.72 mm, N=15) was significantly longer than the control group (1.19$\pm$0.27 mm), N=15, P value = 0.001 (Figure 3).

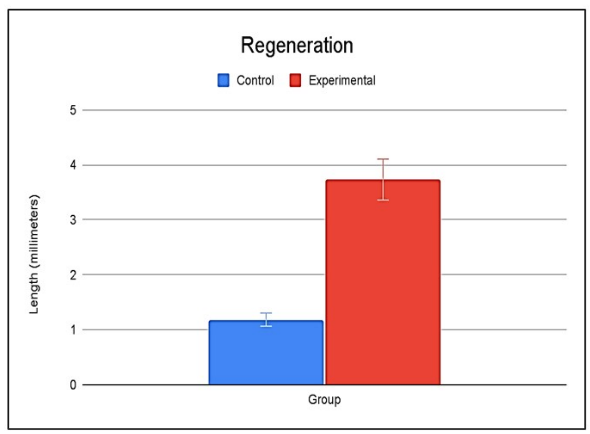

Fig. 3 Regeneration length (mm) of the experimental group (3 groups, N=15) compared to the control group (3 groups, N=15), P = 0001. (Graph by authors, 2023)

There were seven earthworm deaths due to complications after either amputation or electrical stimulation.

Discussion

The aim of the study was to examine the effect of bioelectric stimulation on the bleeding time and regeneration length of earthworms. The results of the study supported the hypothesis that earthworms subjected to electrical stimulation prior to amputation had shorter bleeding time and longer regeneration length compared to earthworms that did not receive electrical stimulation. Results demonstrated no statistically significant difference in the area of blood loss between the experimental group and control group.

In this study, the bleeding time of the earthworms that received electrical stimulation was significantly shorter than that of the control group. The results were congruent to that of Mandel et al. who found the diameter of the femoral artery and vein of rats decreased (vasoconstriction) when exposed to electrical stimulation. At higher voltages, a blood clot formed, and bleeding rate decreased in both the femoral and mesenteric arteries, with hemostasis achieved within seconds[8]. Results were also similar to that of Brinton et al. who noted arterial vasoconstriction during electrical stimulation in rats. The authors found that electrical stimulation constricted the arteries in rats by 41±8% and 37±6% of the initial vessel diameter[9]. The results of the current study support the postulation that vasoconstriction may be induced by neural and non-neural pathways from electrical stimulation causing the blood vessels of the earthworms to decrease in size. This decrease thus slowed down blood flow causing less bleeding time (Figure 4).

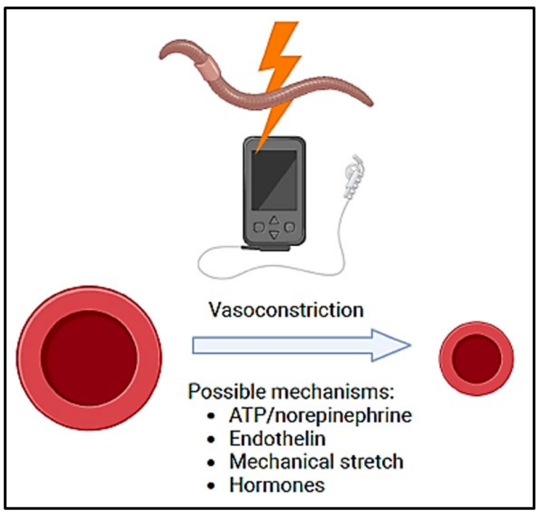

Fig. 4 Proposed mechanism for bioelectrical stimulation causing vasoconstriction in the ventral and dorsal blood vessels (represented by the red circles) of the earthworm (Figure created with Biorender by Authors, 2023)

The current study found no statistically significant difference in the area of blood loss between the experimental group and control group. This is contrary to Mandel et al., who found that the average blood loss from femoral arteries after injury in mice that received electrical stimulation was seven times less than that of the control femoral arteries[8]. The difference in studies may be due to the fact that overlap of blood collected on the blotting paper occurred in the current study. Since area was only measured, the total blood lost from the earthworms may not have been accurate.

Results from the regeneration experiments illustrated that there was a significant difference in regeneration length between the experimental and control groups with the experimental group having a longer regeneration length. The results were consistent with that of Franklin et al. who found that the regeneration process of crawfish was faster using electrical stimulation. Crawfish that received electrical stimulation had a higher percentage (16.35±5.20%) of collagen compared with sham-treated animals (1.47±1.03%, $P < 0.05$)[9]. Similarly, Luo et al, found that electrical stimulation accelerated the regeneration process of human skin wounds[14]. However, investigating regeneration in an animal model of the earthworm, which has innate regenerative properties, is a novel aspect of the current study. After an injury, stem cells activate and regenerate the parts of the amputated segment. When it comes to the differentiation of the stem cells for regeneration, riboflavin plays an important role. Riboflavin is found in the tissue of the worms and comes from gut microbes[11]. It is hypothesized that electrical stimulation leads to more riboflavin production as well as stimulates inflammatory cells, increasing cell proliferation and increasing stem cell differentiation (Figure 5). This proposed mechanism requires further study.

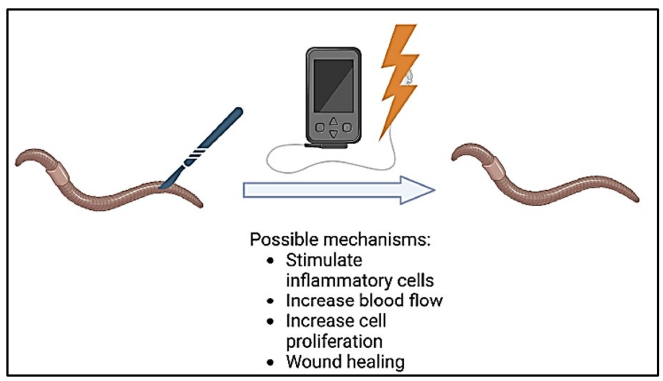

Fig. 5 Proposed mechanism for bioelectrical stimulation accelerating wound healing (Figure created with Biorender by Authors, 2023)

There are limitations to the experiment. As the earthworm is an animal model, the results may not translate to humans. Also, its practical implications on humans are still limited as electrical stimulation can only be administered at certain ranges that will not affect the working of other crucial organs. In addition, this study did not examine mechanisms of how bioelectrical stimulation works in the earthworm. Finally, the use of electrical stimulation to decrease blood flow only increases the healing process and requires alternative methods to control blood flow. However, important insights from this study can be used to study bioelectrical stimulation on humans.

Conclusion

In conclusion, results from this study showed that bleeding time was shorter in earthworms that received electrical stimulation prior to amputation at the eighth segment compared to those who did not receive electrical stimulation. Likewise, the regeneration length in earthworms that received electrical stimulation prior to amputation at the eighth segment compared to those that did not receive electrical stimulation. This study demonstrated that the earthworm serves as a good non-vertebrate animal model to study the effect of bioelectronic stimulation on wound regeneration. In the future, bioelectronic stimulation may be a promising technology to accelerate the recovery of wounds. This technology has important implications for improving wound management in people with chronic medical conditions and those undergoing surgery. Further research is needed in order to understand the mechanisms behind the effect of electrical stimulation on regeneration in earthworms. Future studies can also use different animals with regenerative properties such as planaria. It is also important that in the future different voltage levels and frequencies are tested.

Methods

Design

Earthworms exposed to electrical stimulation, the independent variable, were compared to earthworms who did not receive electrical stimulation. The dependent variables of interest were bleeding time, bleeding area and regeneration length. The experimental group (N=15) was stimulated using a transcutaneous electrical nerve stimulation (TENS) device in distilled water. The control group (N=15) did not receive electrical stimulation. Five earthworms were included in each group and the experiment was repeated three times.

Lumbricus terrestris Culture

Strains of *Lumbricus terrestris* were acquired from Carolina Biological. The earthworms were housed in soil cultures in a refrigerator at less than 60 degrees Fahrenheit. Fox Farm loam soil was used for the soil culture, which was changed every month.

Electrical Stimulation

The experimental group was stimulated using a TENS 7000 machine (Roscoe Inc), while the control group worms were not stimulated. Earthworms were placed in 100 ml of distilled water. The electrodes that connected to the TENS machine were placed into the water such that the metal tips were submerged (Figure 6). The device was set to an intensity of 3 and frequency of 20 Hz. The earthworms were then stimulated for a total of three minutes.

Fig. 6 Electrical Stimulation Set up (Photo by authors)

Amputation of Earthworms

For anesthetization, each earthworm was placed in a beaker filled with 50 ml of carbonated water for five minutes. Immediately after anesthetization, both the experimental and control group worms were amputated using a scalpel at their eighth segments of the head for the bleeding time/area experiments and at the eight segments of the tail for the regeneration experiments. Visual Implant Elastomer (VIE) dye was used to mark the site of amputation (Figure 7).

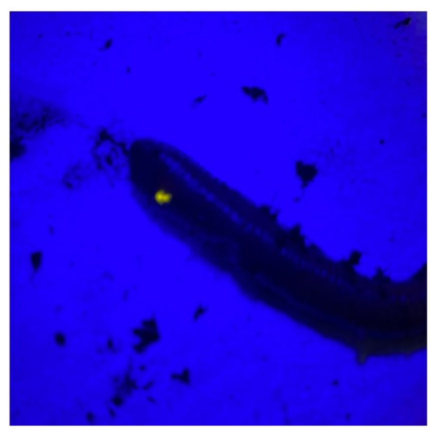

Fig. 7 Visual Implant Elastomer injected worm (Photo by authors)

Bleeding Time of *Lumbricus terrestris*

Bleeding time was measured as the number of minutes it took for the earthworms to stop bleeding after amputation. A paper towel was used to blot the amputated area of the earthworm every 10 seconds. A timer was used to record time. Bleeding was measured as stopped once the paper no longer blotted blood.

Bleeding Area of *Lumbricus terrestris*

Photographs of the blotted paper towel of blood with a ruler were captured for each worm. ImageJ (National Institutes of Health) software was used to measure the area (mm^2) of blood loss.

Regeneration of *Lumbricus terrestris*

The length (mm) of regenerated segments was measured with a ruler from the site of amputation marked with VIE dye for six days for all earthworms.

Data Analysis

Descriptive statistics were used to describe the groups. Means and standard deviations were calculated for bleeding time (min), bleeding area (mm^2) and regeneration length (mm^2) for all groups. Bleeding times and regeneration length of the experimental group and control group were compared using Student's T-test. SPSS 28 (IBM inc) was used and a P value of <0.05 was set as a level of significance.

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