

## Effect of immediate electrical stimulation in the distal segment of the nerve with Wallerian degeneration in rats with sciatic nerve injury

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### ABSTRACT

**BACKGROUND** Electrical stimulation in the proximal segment is one of the modalities for peripheral nerve injury, although it is prone to cause excessive axonal sprouting growth in the proximal segment of the nerve. This study aimed to show that immediate electrical stimulation in the distal segment of the sciatic nerve in Wistar rats accelerated Wallerian degeneration by increasing the expression of tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin (IL)-10, and galectin-3/MAC-2 macrophages to avoid sprouting axons excessively in the proximal segment.

**METHODS** This was an experimental study using male Wistar rats (*Rattus norvegicus*) with a randomized post-test only control group design. The treatment group received immediate electrical stimulation (20 Hz, 2 mA, for 5 sec) to the distal nerve after sciatic nerve injury, while the control group received no treatment. After 3 days, tissue samples were extracted from the distal segment of the sciatic nerve to examine the level of TNF- $\alpha$ , IL-10, and galectin 3/Mac-2 macrophages using ELISA and from proximal nerves to histologically examine the sprouting axons.

**RESULTS** Rats in the treatment group had higher TNF- $\alpha$  (52.1 [10.32] versus 40.4 [17.71] pg/100 mg,  $p = 0.031$ ) and higher IL-10 (918 [167.6] versus 759 [158.9] pg/ml,  $p = 0.010$ ). Expression of galectin 3/Mac-2 macrophages was similar in both groups (465 [49.5] versus 444 [54.4] pg/100 mg,  $p = 0.247$ ). The number of sprouting axons was lower in the treatment group (2 [IQR 1–2] versus 2.5 [IQR 2–3],  $p = 0.003$ ).

**CONCLUSIONS** Immediate electrical stimulation in the distal segment of the sciatic nerve can accelerate nerve regeneration.

**KEYWORDS** electrical stimulation, sciatic nerve, Wallerian degeneration

Peripheral nerve injury remains a global health problem that often results in dysfunction and residual symptoms despite the implementation of optimal surgical and physiotherapy management. The injured peripheral nerve will naturally undergo regeneration.

In the distal segment of the nerve, a degeneration process called Wallerian degeneration occurs, which involves some factors including Schwann cells, macrophages, and various pro-inflammatory and anti-inflammatory cytokines, and is purposed to

remove debris and dead tissue.<sup>1</sup> Regeneration with the restoration of nerve function occurs following the passing of sprouting axons through scar tissue and into the endoneurial tube in the distal segment of the nerve.<sup>2</sup> This nerve regeneration usually takes a long time and is vulnerable to complications.<sup>3</sup>

Previously, the treatment for peripheral nerve injury, which is electrical stimulation to the proximal nerve segment, has potentially caused excessive axonal sprouting growth in the proximal segment of the nerve. The acceleration of Wallerian degeneration in the distal segment of the nerve will provide an optimal condition for axonal sprouting from the proximal segment, thus preventing excessive sprouting, which may result in a neuroma.<sup>2</sup> Up to this point, the study of electrically stimulating the distal segment of injured peripheral nerves to accelerate Wallerian degeneration has not previously been done.

This study aimed to show that immediate electrical stimulation in the distal segment of the sciatic nerve in Wistar rats can accelerate the Wallerian degeneration process by increasing the expression of tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin (IL)-10, and Galectin-3/MAC-2 macrophages to avoid sprouting axons excessively in the proximal segment of the nerve. Immediate electrical stimulation is defined as one-time direct electrical stimulation applied immediately to the distal segment of the nerve after sciatic nerve transection<sup>3</sup> using a disposable electric simulator VARI-STIM II (Medtronic, USA) device with 20 Hz, 2mA, for 5 sec. The results of this study can be used

as the basis of treatment selection in patients with peripheral nerve injury.

## METHODS

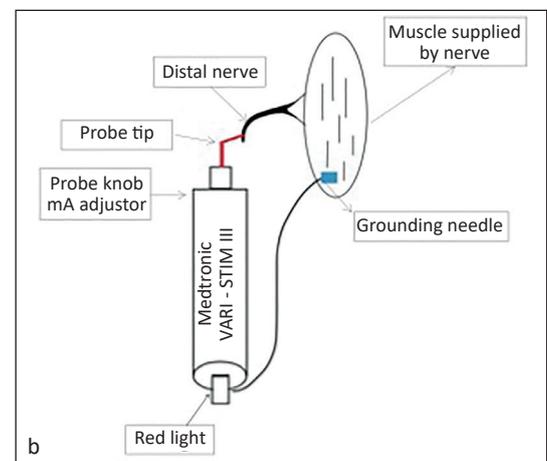
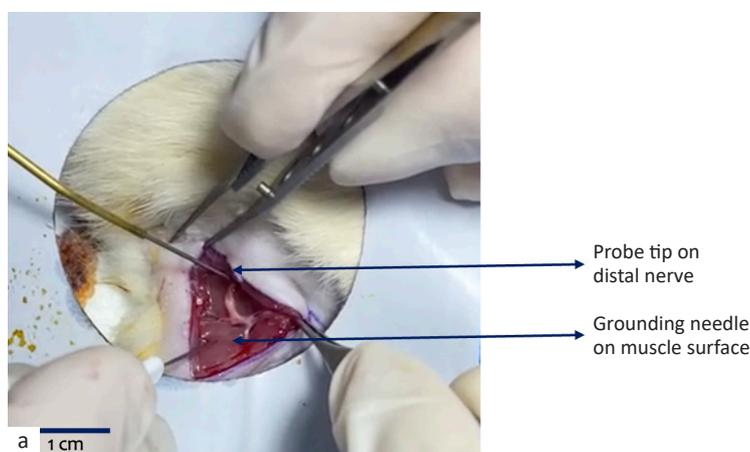
### Study design and sample calculation

This was an experimental study using an animal model with a randomized post-test only control group design. This study was conducted from April to May 2021 at the Integrated Biomedical Laboratory Unit, Faculty of Medicine, Universitas Udayana; Veterinary Pathology Laboratory, Faculty of Veterinary Medicine, Universitas Udayana; and Department of Anatomical Pathology, Faculty of Medicine, Universitas Udayana/Sanglah General Hospital, Denpasar.

### Animals

The experimental animals in this study were male Wistar rats (*Rattus norvegicus*), aged 8–12 weeks (2–3 months), and weighed 150–200 g. Rats were excluded if they were unhealthy (motion inactive) and did not want to eat, which was confirmed by veterinary examination. During the experiment and analysis, no rats were excluded due to illness or any other reasons. A total of 32 rats were divided into two groups. The sample size was determined by Federer's formula, consisting of 16 rats in each group.

Before starting the treatment, all rats in each group were acclimatized for 1 week. Each rat received a different treatment, depending on the randomization. Randomization was obtained by taking odd and even numbers to indicate the treatment and control groups, respectively. The treatment group received



**Figure 1.** Immediate electrical stimulation in the distal sciatic nerve after nerve transection (a) and the schematic figure of the methods (b). The electrical stimulation was conducted using VARI-STIM III (Medtronic, USA). The probe tip was put in the distal nerve while the grounding needle on the muscle surface

immediate electrical stimulation to the distal nerve after sciatic nerve injury (Figure 1), while the control group received no treatment.

### Ethics approval

The experimental protocols were approved by the Ethics Committee of the Faculty of Medicine, Universitas Udayana (No. 132/UN.14.2.2.VII.14/LT/2021) on January 18, 2021. The treatment and maintenance of the animals followed the Guide for the Care and Use of Laboratory Animals by the Animal Care and Use Committee.<sup>4</sup> The cage had a humidity of 55% and a temperature of 25–30°C, controlled with a thermometer. It was made of polyvinyl chloride plastic with dry grain bedding covered with wire and equipped with a feeding tool. Floor mats were changed every 1–2 times a week. The feeding was done every 4 hours with standard food and drink for 24 hours.

### Dissection of the sciatic nerve and electrical stimulation

Anesthesia procedures were performed according to the American Veterinary Medical Association guidelines for the euthanasia of animals.<sup>5</sup> Rats were anesthetized using ketamine (100 mg/ml) 2.5 ml, xylazine (20 mg/ml) 2.5 ml, and acepromazine (10 mg/ml) 1 ml dissolved in 4 ml sterile water (total volume 10 ml) and injected intramuscularly; then, the rats were shaved at the dorsal region of the femur. In the prone position, a skin incision was made at the dorsal region of the femur, and parallel blunt dissection was performed to separate the femur muscle; then, the identification of the sciatic nerve, which was located inferiorly and inside the femur, was performed. Rats in the treatment group had immediate electrical stimulation in the distal nerve right after the sciatic nerve was cut (20 Hz, 2 mA, for 5 sec) (Figure 1). Rats in the control group received no treatment, and the wound was sutured directly after the sciatic nerve was cut; thus, the nerve was allowed to heal naturally.

### Tissue examination

On the 3rd day after the severance of the nerve, re-surgery was performed to take a sample of the nerve tissue for an examination. Tissue sample was harvested on day-3, since a previous study stated that the increase of galectin-3/Mac-2 macrophages was found on day-3.<sup>6</sup> Research materials for the

examination of TNF- $\alpha$ , IL-10, and galectin-3/MAC-2 macrophages were taken from the distal segment of the injured nerve as much as 5 mm under a light microscope.<sup>7</sup> The materials were stored in a cold room with a temperature of –60°C in the Department of Biomolecular until all samples were met. The tissue sample was then extracted and mixed with phosphate-buffered saline (PBS) at a ratio of 30% tissue and 70% PBS fluid 500 ml. The mixture was then centrifuged to obtain serum as the examination material with an ELISA kit.

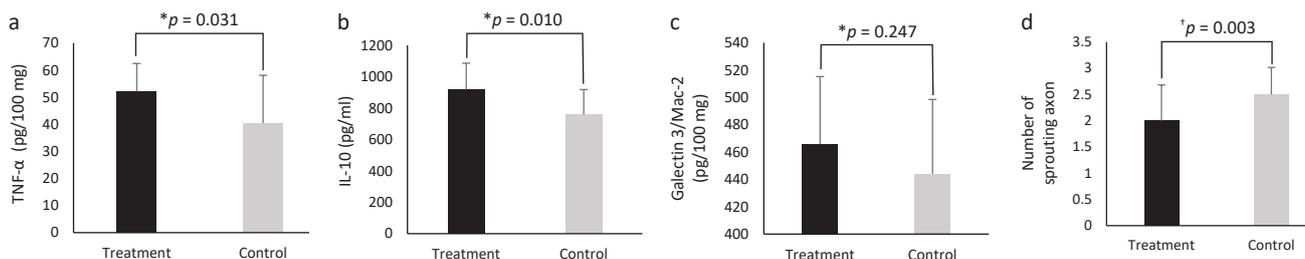
The research material for examining the number of sprouting axons was taken as much as 5 mm from the proximal segment of the transected nerve. The material was fixed in 10% formaldehyde solution, and a histopathological examination was performed. The nerve tissue samples were stained with hematoxylin-eosin, and the number of sprouting axons was counted at 40x and 100x magnifications. To avoid bias, the data were accessed only by one researcher. Other researchers were blinded until the research process was completed.

### Statistical analysis

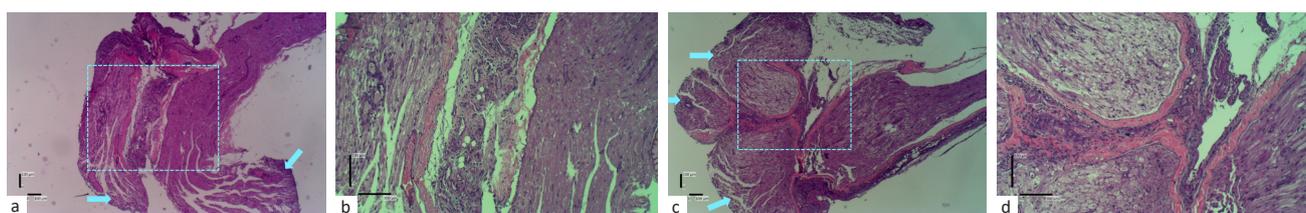
Data collection was entered into the main table using Microsoft Excel 2013 (Microsoft Corporation, USA). The tabulated data were analyzed using SPSS software version 20 (IBM Corp., USA). Macrophages and the number of sprouting axons were tested for normality and homogeneity using Shapiro–Wilk and Levene's tests, respectively. An independent *t*-test was performed with a 5% level of significance ( $p < 0.05$ ) to determine the differences in the levels of TNF- $\alpha$ , IL-10, and galectin/Mac-2 macrophages between both groups. Meanwhile, the Mann–Whitney test was performed with a 5% level of significance ( $p < 0.05$ ) to determine the difference in the number of sprouting axons between both groups.

## RESULTS

A total of 32 male Wistar rats aged 8–12 weeks (2–3 months) and weighing 150–200 g were randomized into treatment and control groups. Figure 2 shows number of sprouting axon was lower in treatment group (2 versus 2.5). Meanwhile, TNF- $\alpha$  (52.1 versus 40.4 pg/100 mg) and IL-10 (918 versus 759 pg/ml) were significantly higher in treatment group, but not for expression of galectin 3/MAC-2 (465 versus 444



**Figure 2.** The level of TNF- $\alpha$  (a), IL-10 (b), Galectin 3/Mac-2 (c), and number of sprouting axons (d) in treatment and control groups. \*Levene's test followed by independent t-test; †Mann-Whitney test. IL=interleukin; TNF- $\alpha$ =tumor necrosis factor-alpha



**Figure 3.** Histopathological sections of axon sprouting. Axon sprouting (indicated by arrows) in the treatment group (a & b) and fewer sprouting axons than in the control group (c & d). Cross-sectional section (a & c) at 40 $\times$  magnification; longitudinal section (b & d) at 100 $\times$  magnification

pg/100 mg). A representative histopathological image of sprouting axons is shown in Figure 3.

## DISCUSSION

This study found an increase in TNF- $\alpha$  and IL-10 levels and a decreasing number of sprouting axons, which are similar to Galectin 3/Mac-2 macrophages in rats receiving electric stimulation in the distal segment. The increase in TNF- $\alpha$  in this study is consistent with an experimental study by Tsaava et al<sup>8</sup> that described an increase in the pro-inflammatory cytokine TNF- $\alpha$  with electrical stimulation for 4 min with a pulse width (50  $\mu$ s, 250  $\mu$ s), amplitude (50  $\mu$ A, 250  $\mu$ A, 750  $\mu$ A), and frequency (30 Hz, 100 Hz) in the cervical vagus nerves of Wistar rats. Similarly, Su et al<sup>9</sup> showed a significant increase in TNF- $\alpha$  expression in the sciatic nerve given transcutaneous electrical nerve stimulation in the proximal part of the nerve immediately after crush injury with high frequency (100 Hz) than with delayed electrical stimulation. However, more significant complications of neuropathic pain were also found.

Local inflammation is the early mechanism of peripheral nerve regeneration, and TNF- $\alpha$  is a pro-inflammatory cytokine expressed mainly in the first phase of Wallerian degeneration, promoting macrophage recruitment from day-2 to -3 after injury. Therefore, increasing TNF- $\alpha$  promotes local

inflammation, which is the early phase of nerve regeneration. After macrophage recruitment, anti-inflammatory cytokines are expressed, preventing excessive inflammatory processes. The anti-inflammatory cytokine in peripheral nerve regeneration is IL-10.<sup>10</sup>

In this study, the expression level of Galectin-3/MAC-2 was not different in the control and treatment groups. A previous study using a rat model with rapid electrical stimulation (10 V/cm, frequency 5 Hz) had successfully induced electrical remodeling, thereby increasing Galectin-3 expression significantly compared with the control group.<sup>11</sup> This finding was supported by Pesheva et al<sup>12</sup> who observed that Galectin-2 and Galectin-3 played a role in the process of axonal regeneration after injury and the pathogenesis of peripheral neuropathy.

Elzinga et al<sup>13</sup> also found that electrical stimulation may induce axonal growth by increasing the expression of Galectin-3/MAC-2 and other neurotrophic factors such as glial cell-derived neurotrophic factor and brain-derived neurotrophic factor. Galectin-3 is a molecule that plays a role in microglial phagocytosis through Fc $\gamma$ R and CR3/MAC-1, and it targets the cytoskeleton during nerve injury. Moreover, studies have found that Galectin-3 accelerates Wallerian degeneration by modifying the toll-like receptor and expression of pro-inflammatory

cytokines in the injured sciatic nerve.<sup>14</sup> This is related to the role of GAL-3 and GAL-1 in inducing neuronal repair and preventing axonal loss.<sup>15</sup>

Atkins et al<sup>16</sup> showed that low concentrations of IL-10 could decrease scar formation following sciatic nerve injury and trigger axon regeneration. This can be triggered by electrical stimulation in the distal injury area, which produces low concentrations of IL-10, thereby increasing axon fiber regeneration. Low dosages of IL-10 increase the number of axons in the proximal segment of the nerve. A scar can arise due to a mechanical barrier to axon sprouting produced by the formation of a neuroma at the axon terminal. The results demonstrated that cytokine IL-10 effectively reduced scar formation and increased regeneration in peripheral nerves.<sup>17</sup>

In this study, electrical stimulation was given immediately for 5 sec (20 Hz, 2 mA) in the distal segment of the injured nerve. As seen in the results, no significant Galectin-3/MAC-2 was shown. This finding shows that multiple stimulations with various durations and frequencies, as well as harvesting the tissue on different days, may elicit significant results. An animal study by Samiee et al<sup>18</sup> showed that the electrical stimulation in the sciatic nerve accelerated nerve repair and indirectly improved biceps femoris muscle force to a comparable level with control without effecting muscle sensitivity. In that study, the rat's sciatic nerve was subjected to daily electrical stimulation for 2 weeks (duration: 0.2 sec, frequency: 100 Hz, amplitude: 15 mA). Electromyography (EMG) was recorded from the biceps femoris and gluteus maximus muscles on day-3, -7, -10, and -14 after sciatic nerve ligation. Muscle strength and sensitivity were determined by processing the recorded EMG signals in the respective time and frequency domains. Further analysis showed no significant difference between the nerve groups ligated without electrical stimulation and with electrical stimulation on day-3, -7, and -10 after ligation. However, the main difference occurred on the 14th day after the administration of electrical stimulation.<sup>17</sup>

Early in injury, macrophages contribute by producing TNF- $\alpha$  and IL-1 $\beta$ . Galectin-3/MAC-2 macrophages are a family of lectins that are frequently present in the nucleus and cytoplasm of many cells. Galectin-3/MAC-2 macrophages in the cytoplasm activate myelin phagocytosis via macrophages and microglia. M1 macrophages were found on the 1st

and 3rd days after injury, but M2 macrophages were found on the 3rd day and persisted until day-14. It has been suggested that M2 macrophages regulate the inflammatory response in Wallerian degeneration, and the regeneration of nerves can occur in parallel or alternately.<sup>18</sup> Sprouting axons originate in the terminal nodes of Ranvier, formed shortly after peripheral nerve trauma and extended to the surface of the Schwann cells or the interior of the basal lamina. When the proximal and distal stumps coincide, the growth and branching of axons can provide a framework for nerve fusion. In the regeneration process, sprout axons are expected to grow in a single columnar fiber and undergo myelination.<sup>19</sup> If the regeneration of sprouting axons does not reach the distal stump, it will spread and become a neuroma. Therefore, a proper regulation of cytokines is needed to control axon growth.<sup>19</sup>

In this study, the median number of sprouting axons in the treatment group was lower than the control group (median 2 versus 2.5,  $p < 0.05$ ). Nerves that are given with immediate electrical stimulation have more controlled axon growth, which prevent the occurrence of neuromas.<sup>20</sup> Axon growth is known to be regulated by a chemotactic signal. According to Atkins et al,<sup>16</sup> increased expression of IL-10 also affects axon regeneration in peripheral nerves. An IL-10 injection was administered to the injured nerve, and the number of myelinated axons was measured proximally and distally. The results showed an increased number of myelinated axons. The study also found a low amount of collagen type I and III, thereby preventing excessive axon branching that causes neuromas.<sup>17</sup>

The limitation of this study is the administration of electrical stimulation that was carried out with a frequency of 20 Hz for 5 sec immediately after the termination of the sciatic nerve. However, it would be necessary to provide a stimulation with different frequencies and durations and investigate the administration of delayed electric stimulation to determine its effect on the process of nerve regeneration.

In conclusion, immediate electrical stimulation in the distal segment of the nerve after sciatic nerve transection can accelerate nerve regeneration by increasing the expression of TNF- $\alpha$  and IL-10 and decreasing the number of sprouting axons. These results can be used as a basis for further study in

humans to select the method of nerve stimulation in patients with peripheral nerve injury.

#### Conflict of Interest

The authors affirm no conflict of interest in this study.

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