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PRS GLOBAL OPEN

ORIGINAL ARTICLE
Hand/Peripheral Nerve

Negative Pressure Neurogenesis: A Novel Approach to Accelerate Nerve Regeneration after Complete Peripheral Nerve Transection

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Background: Various modalities to facilitate nerve regeneration have been described in the literature with limited success. We hypothesized that negative pressure applied to a sectioned peripheral nerve would enhance nerve regeneration by promoting angiogenesis and axonal lengthening.

Methods: Wistar rats' sciatic nerves were cut (creating ~7 mm nerve gap) and placed into a silicone T-tube, to which negative pressure was applied. The rats were divided into 4 groups: control (no pressure), group A (low pressure: 10 mm Hg), group B (medium pressure: 20/30 mm Hg) and group C (high pressure: 50/70 mm Hg). The nerve segments were retrieved after 7 days for gross and histological analysis.

Results: In total, 22 rats completed the study. The control group showed insignificant nerve growth, whereas the 3 negative pressure groups showed nerve growth and nerve gap reduction. The true nerve growth was highest in group A (median: 3.54 mm) compared to group B, C, and control (medians: 1.19 mm, 1.3 mm, and 0.35 mm); however, only group A was found to be significantly different to the control group (**$P < 0.01$). Similarly, angiogenesis was observed to be significantly greater in group A (**$P < 0.01$) in comparison to the control.

Conclusions: Negative pressure stimulated nerve lengthening and angiogenesis within an in vivo rat model. Low negative pressure (10 mm Hg) provided superior results over the higher negative pressure groups and the control, favoring axonal growth. Further studies are required with greater number of rats and longer recovery time to assess the functional outcome. *(Plast Reconstr Surg Glob Open 2021;9:e3568; doi: 10.1097/GOX.0000000000003568; Published online 13 May 2021.)*

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Received for publication February 14, 2021; accepted February 18, 2021.

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DOI: 10.1097/GOX.0000000000003568

INTRODUCTION

Peripheral nerve injuries are relatively common, often occurring through diverse types of traumatic events, such as motor vehicle accidents, and can lead to long-term disability, pain, and financial burden, thereby contributing to an individual's reduced quality of life.[1-4] Although injured peripheral nerves can regenerate, axon regeneration proceeds slowly, at rates of only 1–3 mm/day.[2,5,6] No definitive therapeutic methods have been devised to speed this rate of regeneration.[7] The regenerative capacity of axons and the growth support of Schwann cells decline with time and distance from injury.[2]

Various modalities to facilitate nerve regeneration—such as neurotrophic factors—have been described in the literature with limited success.[7–26] There have been limited reports of applying electrical fields/gradients across a repaired peripheral nerve to speed up axonal regeneration.[27-31] However, the mechanisms by which electrical stimulation enhances nerve regeneration remain relatively poorly understood, and the misdirection of regenerating axons after surgical repair remains a problem for the appropriate activation of re-innervated muscles.[32]

Disclosure: *All the authors have no financial interest in relation to the content of this article.*

Nerve guidance conduits have drawn attention as an aid to promote the regeneration of injured axons across damaged peripheral nerves.[33,34] Neuronal cells exposed to mechanical stretch stimulated by 10% equibiaxial strain at 0.25 Hz showed neurite outgrowth (both length and number).[35] Distraction neurogenesis with an experimental nerve-lengthening device was successful for the reconstruction of nerve defects of 10 mm in Sprague Dawley rats. However, the potential drawbacks would be the difficult therapeutic application and the device-related problems, like mechanical failure and infection.[36] The possibility of axon stretching in vitro was explored, where rat dorsal root ganglia neurons were grown on 2 adjoining substrates. The bridging axons were allowed to grow across the interface and into the adjacent population of neurons.

Axons were stretched by displacing the 2 adjoining substrates to achieve stretch growth rates up to 10 mm/day. This concept is significantly more challenging in vivo though, due to the technical difficulties of applying mechanical forces to the axons without inflicting wider tissue damage.[37]

Negative pressure therapy is an established method for promoting tissue healing. Multiple mechanisms are postulated to be responsible for this effect: removal of excess interstitial fluid decreases localized edema and increases local blood flow, which decreases bacteria levels in tissue, while mechanical deformation of cells results in an increase of the rate of cell proliferation and in protein matrix synthesis.[38] Based on the effect of negative pressure wound therapy, in vitro axonal stretching, the principle of nerve elongation during embryological development, and limb lengthening procedures, we hypothesized that by applying negative pressure to the proximal end of a newly transected peripheral nerve (in a rat model) that would enhance axonal elongation/regeneration compared with the control (no negative pressure).

MATERIALS AND METHODS

A total of 30 adult Wistar rats aged 3 months with an average weight 450 g, were approved by the university's Ethical Committee (NRS/01/17/AEC) for the study to examine the effect of negative pressure on transected sciatic nerves. As the ethics committee considered this pilot study novel with potential for adverse outcomes on the animals, it approved only a small number of animals over a single time-point (1 week only). Figure 1 shows the flow chart study design. We started with the control (no negative pressure) and low-pressure groups to ensure the pressure was tolerated by the animal without distress. The subsequent groups were allocated to the 2 higher negative pressure groups. The sciatic nerve for an adult Wistar rat is approximately 1 mm in diameter. We used a custom-made transparent silicone T-tube with 1.5 mm inner diameter for our study. The left sciatic nerve of each rat was cut and placed into a silicone T-tube (with ~7 mm gap) to which negative pressure was applied using a customized portable suction device with digital pressure monitor.[39] The rats were divided into 4 groups: control (no pressure), low (−10 mm Hg), medium (−20/30 mm Hg) and high (−50/70 mm Hg). The rats were monitored continuously via cameras to ensure their welfare while the negative pressure was recorded to ensure stability with an allowed fluctuation of ±2 mm Hg. After recovery from anesthesia, the rats were free to move within their cages and euthanized at 7 days post-surgery. On day 7, the nerve segments were retrieved for gross and histological analysis.

Surgical Procedure

Surgery was performed on the left sciatic nerve. The T-tube was sterilized with 100% ethanol and flushed with sterile normal saline. The rats were anaesthetized with O2/Isoflurane mixture (30%/1%–3%). Surgical sites were shaved away from the surgical field at both the left gluteal region (primary surgical site) and the back of the cervical region (the exit point for tubing; secondary surgical site). Rats were placed prone over a heat blanket and limb stabilization was achieved via an adhesive tape. Buprenorphine (0.05 mg/kg subcutaneously) was administered intra-surgically and post-surgically. Both surgical sites were cleaned and treated with alcoholic iodine.

A skin incision was performed extending from a midpoint (between the hip joint and ischial tuberosity) to the knee. Blunt dissection was carried out (muscle splitting approach) using Iris scissors between the gluteus maximus and biceps femoris muscle. The sciatic nerve was identified under the gluteus maximus muscles. The nerve was isolated from the surrounding connective tissues and fascia using micro-scissors. The epineurium and its blood vessels were preserved. The position of the T-tube was checked for tunnel planning. Subcutaneous tunneling was performed in 2 steps with the use of a "passing probe." Suspensory skin sutures were used (~5 cm proximal to the initial skin incision) to stabilize the tube.

Sciatic Nerve Transection and Implantation of T-tube

The nerve was transected with sharp micro-scissors (in the middle of the exposed length of the nerve). The T-tube was tunneled under the gluteal muscles in the primary surgical site and then subcutaneously superficial to the back muscles. The long limb of the T-tube emerged from a small skin portal behind the neck (secondary surgical site) to provide a safe portal away from the rat's mouth with no restriction of mobility.

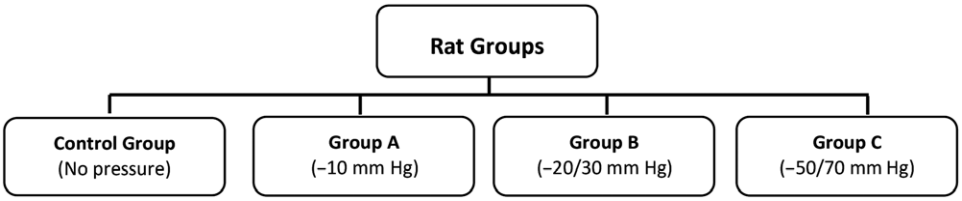

Fig. 1. Flow chart showing study design.

The sectioned nerve ends were fed through the 2 limbs of the T-tube using negative pressure to minimize nerve handling with surgical instruments. Once the nerve ends were fed into the tube, the negative pressure was removed, and two 8/0 nylon sutures were used to secure the nerve end to the silicone tube. We used surgical skin glue (HistoAcryl from B Braun) to secure the tube to the underlying muscles to restrict the rotational moment of the tube while the rat is moving and hence prevention of the nerve end dislodgement from the tube. The average proximal nerve end in the tube was 4.2 mm, whereas the distal nerve end was 3 mm. The nerve gap was measured in millimeters (average 7.1 ± 2.9 mm) between the proximal, and distal ends of the nerve inside the tube. The nerve end was well sealed and no additional sealant around the nerve was required.

The wound was flushed with sterile normal saline. The skin was sutured with 6/0 PDS (absorbable suture). Surgical Opsite spray (Smith & Nephew) was applied to the wound to keep wound sterility and prevent contamination. The long limb of the T-tube was secured to the back of the rat with nylon suture and fed through the harness swivel system for extra security. The connector silicone tubing system from the harness to the upper cage was protected with an outer metal spring to prevent the rat from biting the tube. The connector tube was connected to the top of the cage through a hollow swivel to transmit the negative pressure and permit rat mobility (Fig. 2).

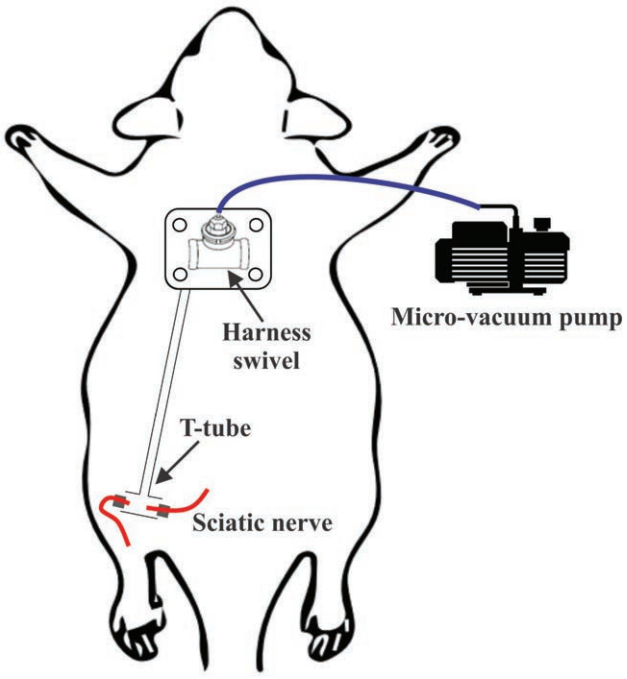

Fig. 2. Schematic diagram showing the design of the surgical procedure, red lines represent the 2 ends of the sciatic nerve after transection and insertion into the transverse limb of the T-tube. The longitudinal limb of the T-tube is tunneled subcutaneous to emerge behind the rat's neck and is attached to the swivel harness system through which the negative pressure is introduced.

Postoperative Care

Negative pressure was applied to the nerve ends continuously for 7 days and set to the respective group pressure value.[40] Postoperatively, the rats recovered in their cages. The rats were closely monitored after the procedure for any adverse effects. Daily checks on the rats' activity and wellbeing were carried out according to the ethics approved monitoring sheet.

Nerve Retrieval and Pathological Testing

At the seventh postsurgical day, the rats were anaesthetized and the nerve ends within the T-tube were retrieved. The rat was euthanized by intra-peritoneal Lethabarb injection concurrent with isoflurane. Lengths of each end of the nerve within the tube were recorded before harvesting. The retrieved nerve ends were fixed in 4% paraformaldehyde for 2–4 hours then washed in 0.2% glycine in phosphate-buffered saline (PBS). Nerve ends were transferred in 70% EtOH. The nerve ends were treated with osmium tetroxide (OsO4) for myelin sheath visualization then processed and embedded into paraffin wax for sectioning.

Six transverse sections (thickness: 5 μm) were collected at 250-μm intervals along the entire length of the nerve. These intervals ensured that different regions along the regenerating nerve were analyzed. One nerve slice per section was counter stained with Masson Trichrome to provide connective tissue and blood vessel analysis.

Statistical Analysis

The statistical analysis of the experimental data was performed either using parametric or nonparametric ANOVA (Kruskal-Wallis test also known as ANOVA by ranks) based on the outcome of normality test (Shapiro-Wilk test) to observe the effect of negative pressure on length of nerve zones, gross and actual nerve length, and angiogenesis. This was followed by post hoc test (Dunnett's or Tukey's) to compare means or medians between 2 independent groups to ascertain which treatment group was significantly different than the control. Statistical analyses were performed using GraphPad Prism 8.4.0 (GraphPad Software, San Diego, Calif.). For every analysis, null and alternative hypotheses were tested. The null hypothesis (Ho) assumes that there is no difference between the observed value and the control, and the results are random due to chance. The alternate hypothesis (Ha) says that the results are because of treatment (negative pressure effect) and are not due to chance. To reject a null hypothesis, differences among control and treatment groups were considered significant at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

RESULTS AND ANALYSIS

All rats tolerated the surgical procedure and negative pressure well, except 1 animal, which did not recover from the anesthesia. No animals displayed any signs of distress after surgery. It was noted that the nerve stumps of 7 rats were dislodged from their respective T-tubes, which was evident from the pressure graphs (these events were before using the surgical glue to stabilize the tube to the

Table 1. Total Rats Included in the Analysis

Groups	No. Rats
Control	4
Group A (low pressure: 10 mm Hg negative pressure)	4
Group B (medium pressure: 20/30 mm Hg negative pressure)	7
Group C (high pressure: 50/70 mm Hg negative pressure)	7
Totals rats included in results/analysis	22

surrounding tissues). These 7 rats were removed from further analysis. The number of rats in each group that were included in analysis is provided in Table 1.

Measurement of Nerve Growth

No reduction in the nerve gap was found in the control group, whereas a variable gap reduction was noted in the 3 treatment groups. This reduction in the nerve gap was due to the lengthening of the proximal nerve stump within the T-tube. Our findings coincided with previous understanding that a nerve regenerates from the proximal stump. The apparent increase in the length of the proximal nerve end inside the tube was found to be (median with IQR) 3.9 (1.175) mm for group A; 2.45 (0.45) mm for group B, 2.25 (0.725) mm for group C, and 0.6 (0.3) mm for the control group (See Table 2). These results showed that the highest difference in the apparent length of the proximal nerve stump inside the tube was in group A followed by group B and then group C, whereas the control group showed the lowest difference. Statistical analysis of the data followed by Dunn's multiple comparisons test confirmed that only groups A and B are statistically significantly different than the control group (***$P < 0.001$, *$P = 0.032$).

Division of the Proximal Nerve into Zones

The Masson Trichrome stained sections at 250 µm intervals along the nerve were examined by a blinded medical pathologist who classified the retrieved nerve ends into 3 regional zones—as described below—based on qualitative pathology and nerve anatomical morphology (Figs. 3–5). The morphology of the nerve in Zone 1 was interpreted to be granulation tissue, which was disparate to the other 2 zones; hence, representative sections of Zone 1 were further stained with Haematoxylin and Eosin for validation.

Zone 1 is the zone of transection. Microscopic analysis of this region revealed the presence of cellular debris, neutrophils, lymphocytes as well as foamy macrophages. Some macrophages have myelin debris (stained black with Osmium). Vascular regeneration and fibroblasts with connective tissue were observed. The length of this zone varied between controls and some of the pressure groups.

Zone 2 zone proximal to the transection. Fibrotic endoneurium, scarce epineurium, and perineurium were seen along with the presence of some intact myelinated axons.

Zone 3 is the zone proximal to Zone 2, which represents myelinated native nerve fibres and regenerating nerve. The number of myelinated axons gradually increased (qualitatively assessed) with increasing distance away from transection and intact endoneurium, epineurium and perineurium were observed.

Table 2. Measurement of the Length of Proximal Nerve End Inside the T-tube (in Millimeters)

Rat Groups	Length of Nerve Stump at the Day of Surgery—Day 0 (Li)*	Length of Nerve Stump at the day of retrieval — Day 7 (Lf)*	Lf – Li Apparent Increase in Nerve Lengths*	Median, IQR (Q3-Q1)
Control	3.8 ± 0.8	4.4 ± 1.4	0.6 ± 0.15	0.6, 0.3
Group A	3.75 ± 1.2	7.95 ± 2	4.2 ± 0.91	3.9, 1.175
Group B	4.7 ± 2.3	7.2 ± 1.5	2.5 ± 0.25	2.45, 0.45
Group C	4.1 ± 0.9	6.3 ± 1.1	2.2 ± 0.33	2.25, 0.725

*Measurements in millimeters (Mean ± SD) of proximal nerve end.

DISCUSSION

Length of Proximal Nerve Zones

The average length of each zone for each group was calculated from the histological analysis (Fig. 5). Zone 1 (zone of granulation connective tissue) showed a positive correlation with pressure, as demonstrated by an increase in length relative to an increase in the negative pressure up to 30 mm Hg; however, none of the groups were found to be significantly different to the control, with median and interquartile range of 0.29 (2.09) mm, 1.11 (3.38) mm, 0.74 (1.06) mm, and 0.45 (0.4) mm for group A, B, C, and control respectively. Similarly, Zone 2 (degeneration/regeneration zone) did not show any significant difference among the 4 groups ($P > 0.05$) with median and interquartile range of 1.65 (0.95) mm, 1.66 (0.485) mm, 1.60 (0.38) mm, and 1.10 (0.98) mm, for group A, B, C, and control, respectively. In contrast, Zone 3 (myelinated growing nerve/native nerve zone) showed an increase in length in the 3 negative pressure groups when compared with the control. Median length of the zone 3 was found to be 5.38 mm (IQR: 2.56) in group A, 3.4 mm in group B (IQR: 2.86), 3.54 mm in group C (IQR: 1.16), and 2.8 mm

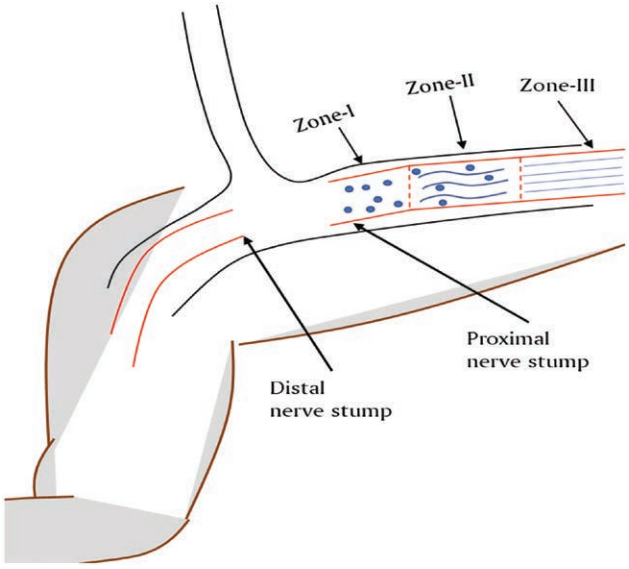

Fig. 3. Schematic diagram showing the 3 zones of the proximal nerve end inside the tube.

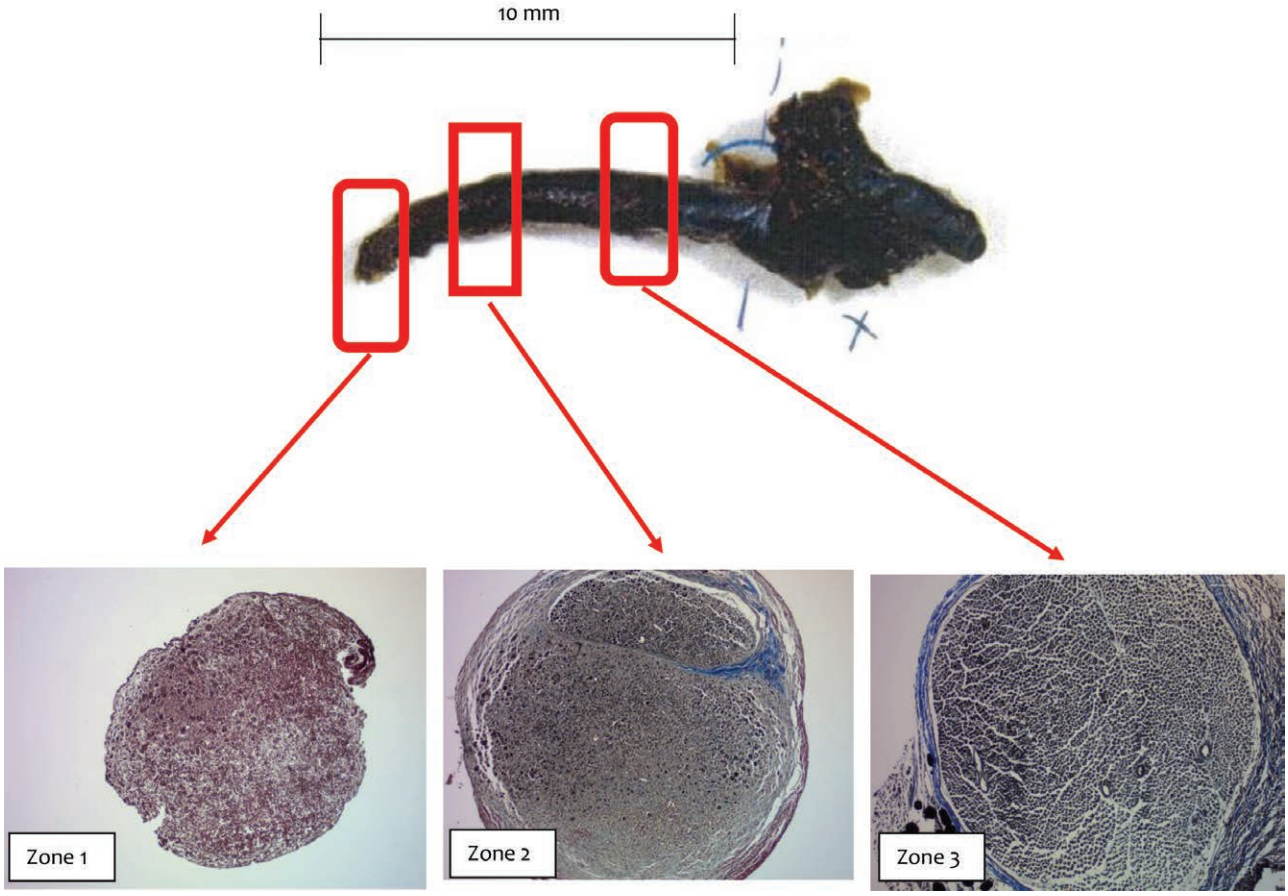

Fig. 4. Sample of a nerve fixed with Osmium and counter stained with Masson Trichrome, displaying the 3 distinct zones. Myelin sheath visualization (stained black) appears as black rings, whereas degenerating myelinated fibres appear disordered (black blobs). Mason Trichrome provides connective tissue visualization (collagenous tissue stained blue, while cellular nuclei stained dark red/purple, and the cytoplasm red/pink). The healthy myelin sheath was found in Zone 3 and, to a lesser extent, in Zone 2, whereas Zone 1 has myelin debris.

(IQR: 1.8) in the control group. However, only group A was found to be statistically significantly different to the control group ($P < 0.05$). We also noted a negative correlation in the length of zone 3 with increasing the negative pressure value.

Nerve Growth Metrics

On gross examination (day 7), there was no significant nerve growth in the control group, whereas the 3 treatment groups showed increased gross nerve growth, which reflected a reduction in the nerve gap within the tube. As the leading end of the proximal nerve represents connective tissue (zone 1) and not true nerve regeneration, we subtracted the length of nerve in zone 1 to estimate the true length of nerve regeneration. Subtracting the connective tissue zone—determined by the histological analysis—we found that the true nerve growth (Fig. 6) was highest in the low- negative pressure group (group A). The true growth was found to be (median length with IQR range) 3.54 (2.12) mm, 1.19 (2.4) mm, 1.3 (0.682) mm, and 0.35 (0.4) mm for groups A, B, C, and control, respectively. Dunn's multiple comparison test confirmed that only group A was statistically significantly different to the control group ($^{**}P < 0.01$).

Angiogenesis Analysis

The number of blood vessels from the Masson Trichrome stained slides (using 100× magnification) was calculated in zone 2 of each group as representative of angiogenesis (Fig. 7). The data were analyzed using nonparametric analysis of variance (Kruskal-Wallis Test), which indicated a significant variation among the medians of control and treatment groups ($P = 0.0003$). Angiogenesis was found to increase in all 3 negative pressure groups in comparison with the control (Fig. 7B) with a median (+IQR) of 24.5 (4.5), 18 (6), 15 (3.5), and 14 (3) blood vessels in group A, B, C, and control, respectively. Multiple comparison test (Dunnett's post-hoc test) confirmed that only group A was significantly different than the control ($^{**}P < 0.01$). This analysis suggested that low level of negative pressure positively affects the formation of vascular bundles during nerve growth than the control and other treatment groups (groups B and C). We also explored the distal stumps of the transected nerves but found no difference among the groups.

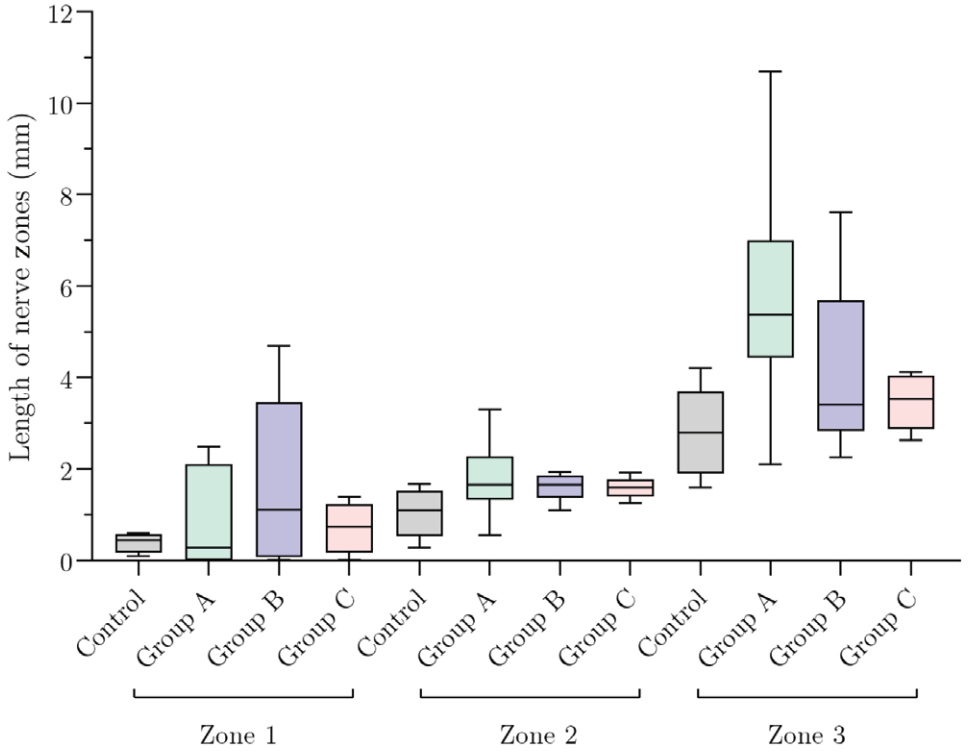

Fig. 5. Length of nerve zones.

Limitations and Conclusions

This novel study had a number of limitations. The study design was based on an anticipated effect size 1.5 greater than the control. No in vivo pressure guidance was available before our study and the possible deleterious effect was unknown. Therefore, small animal cohorts and a short experimental duration were specified from the University's animal ethics committee. The number of rats was not equal in each group as 1 rat died during anesthesia, and seven rats were excluded from the study because of dislodgement of the nerve end from the tube. We modified our surgical technique to overcome the latter problem by using suspensory skin sutures, Histacryl glue to secure the tube to the muscle and changing the position of the subcutaneous tunneling. It was not possible to predict an obstruction of the tube despite continuous monitoring due to the small size of the tube's inner diameter (1.5 mm). Because our study was conducted for 7 days only, we could not follow the progression of the regenerating axons over a longer duration.

We conclude that low negative pressure (10 mm Hg) favored angiogenesis over the control group and provided superior axonal growth over the higher negative pressure groups and the control. This increase in the length of the proximal nerve ends in the low pressure group was represented by a significant growth through zone 3 (myelinated growing nerve/native zone). We believe that true regenerative growth occurred with early sprouting and macro deformation facilitated by the negative pressure effect. This growth could not reflect stretch of the nerve, as the growth was the highest in the low negative pressure group and the lowest in the high negative pressure group. Also, the proximal nerve end was secured with sutures (8/0 nylon) to the tube; this would make it very unlikely that the nerve end was drawn into the tube creating a false increase in its length.

We believe that Zone 1 represents the connective tissue scaffold into which the sprouting axons will grow. We hypothesize that negative pressure applied to the proximal

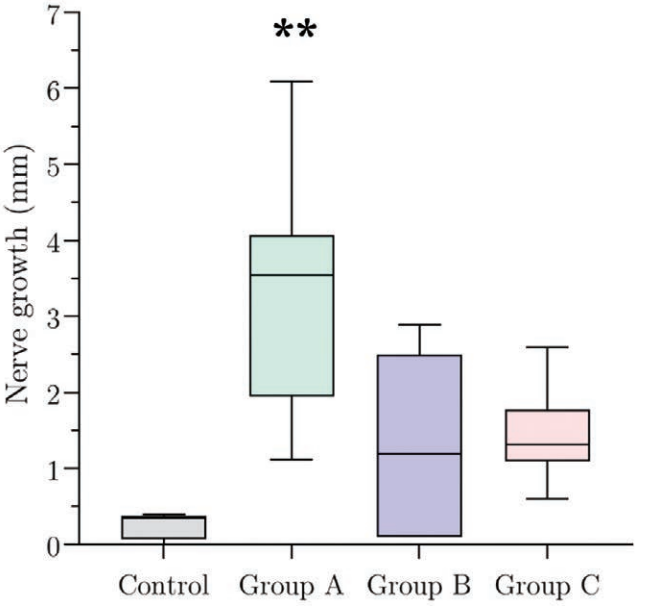

Fig. 6. True nerve growth in millimeters (group A was statistically significant $^{**}P < 0.01$).

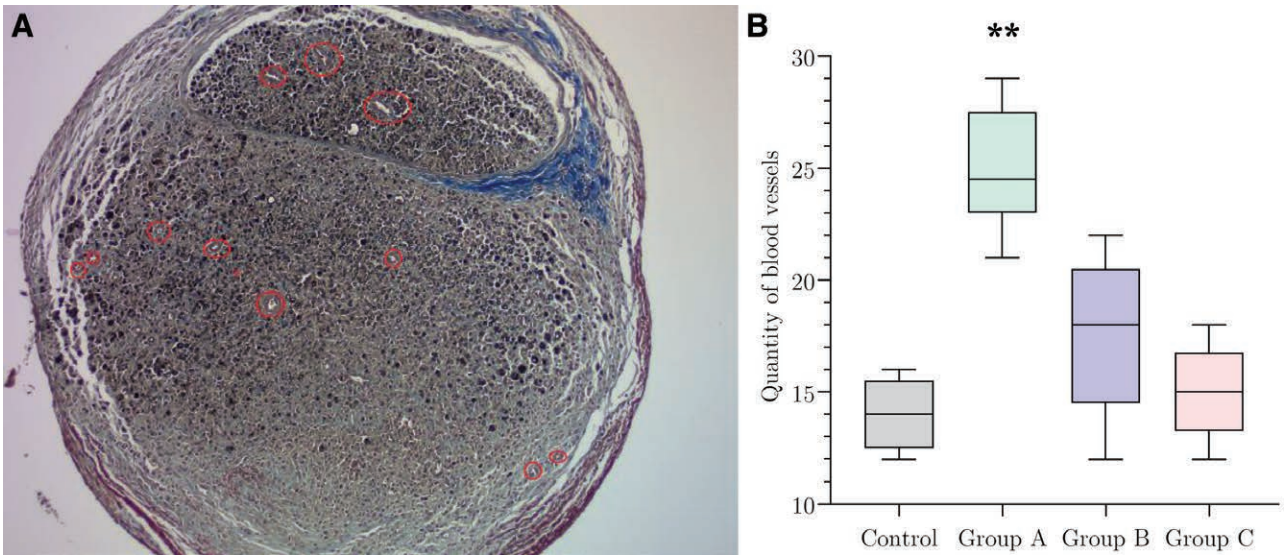

Fig. 7. A, Counting blood vessels in Zone 2. B, Quantity of blood vessels in each group. Group A was statistically significant when compared to the control (**$P < 0.01$).

Table 3. Possible Effects of Controlled Negative Pressure on Neurogenesis

Direct Effects	Indirect Effects
Draw more fluid from neighboring blood vessels in the nerve's epineurium to support growing neurites of axons at the proximal stump.	Faster regrowth rate of regenerating axons
Direct the growing neurites in a confined tube and guide them toward the distal end.	Mitigate the risk of aberrant axonal sprouting
Macro-deformation stretching nerve fibers (axon elongation), unidirectionally to increase the nerve regrowth rate.	Allows a lengthening (true growth) of the native nerve

end of a transected peripheral nerve would enhance nerve regeneration by different mechanisms (Table 3).

This pilot study has demonstrated that negative pressure stimulated the nerve growth in a rat sciatic nerve gap model. This study is clinically translatable by combining the known beneficial effects of negative pressure on tissue regeneration and proven efficacy of conduit nerve repair. The technology would be useful for augmenting nerve regeneration in situations where primary nerve repair outcomes are poor, such as proximal nerve injuries and nerve gap repair.

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ACKNOWLEDGMENT

This work was supported by a grant from the Australia Orthopaedic Association (AOA) and Queensland Hand Surgery Society (QHSS). This study was approved by (NRS/01/17/AEC) and conducted at Griffith University, Queensland, Australia.

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