

# Successful Reconstruction of Nerve Defects Using Distraction Neurogenesis with a New Experimental Device

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## ABSTRACT

**Introduction:** Repair of peripheral nerve injuries is an intensive area of challenge and research in modern reconstructive microsurgery. Intensive research is being carried out to develop effective alternatives to the standard nerve autografting, avoiding its drawbacks. The aim of the study was to evaluate the effectiveness of a newly designed mechanical device for the reconstruction of the sciatic nerve in rats in comparison to nerve autografting and to assess the pain during the period of distraction neurogenesis.

**Methods:** Fourteen Sprague Dawley rats were used and randomly assigned into 2 groups with 7 rats in each group; group A (Nerve Autografting group) in which a 10-mm segment of the sciatic nerve was resected and rotated 180 degrees, then primary end-to-end neurorrhaphy was performed in the reverse direction; group B (Nerve Lengthening group) in which the mechanical device was inserted after surgical resection of 10 mm of the sciatic nerve, then secondary end-to-end neurorrhaphy was performed after completing the nerve lengthening. Thirteen weeks later, assessment of the functional sciatic nerve recovery using static sciatic index (SSI) was performed. Furthermore, fourteen weeks after the nerve resection, assessment of the nerve regeneration with electrophysiological study and histological analysis were performed. Also, gastrocnemius wet weight was measured. For pain assessment in group B, Rat Grimace Scale (RGS) score was used.

**Results:** Significantly better functional recovery rate (using the SSI) was reported in the nerve lengthening group in comparison to autografting group. Also, a statistically significant higher nerve conduction velocity was detected in the nerve lengthening group. On histological analysis of the distal nerve section at 3 mm distal to the nerve repair site, significant myelin sheath thickness was detected in the nerve lengthening group.

**Discussion:** Distraction neurogenesis with the new experimental device is a reliable therapeutic method for the reconstruction of nerve defects.

### Key Words:

Nerve lengthening,  
Peripheral nerve injuries,  
Nerve lengthening,  
Nerve regeneration

## 1. Introduction

Peripheral nerve lesion is a common injury (Johnson & Soucacos, 2008) with high incidence in crushed injuries of the extremities (Taylor, Braza, Rice, & Dillingham, 2008).

Repair of peripheral nerve injuries is an intensive area of challenge and research in modern reconstructive microsurgery. Despite the remarkable development and innovation in microsurgical techniques and instruments, a satisfactory functional outcome is rarely achieved after peripheral nerve repair (Wood, Kemp, Weber, Borschel,

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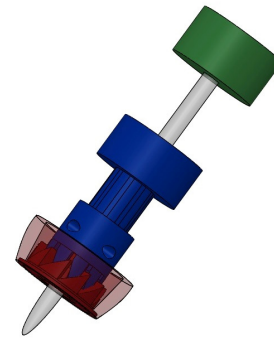
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& Gordon, 2011). Peripheral nerve injury is associated with significant and permanent morbidity and as a result, a considerable medical and economic burden for both the patient and the community (Siemionow et al., 2011). Whenever applicable, end-to-end neurorrhaphy would achieve the best results, however in case of crushed nerve injuries or surgical nerve resection during tumor excision, a nerve defect develops. Autogenous nerve grafting is considered the gold standard treatment to bridge a nerve defect that allows tension free end-to-end repair and provides the neurotrophic factors for nerve regeneration (Millesi, 1998). However, there are several drawbacks of its use such as limited availability in large defects, discrepancy of diameter between the nerve graft and nerve stumps, avascularity of the grafted tissue, and harvest site morbidity with neuroma formation and sensory loss besides disappointing functional recovery in the reconstruction of large nerve defects that might be due to the fact that regenerating axons have to cross two anastomotic sites, hence decrease the chance to arrive their target (re-innervated) tissue (Krarup, Archibald, & Madison, 2002).

In order to avoid such drawbacks, intensive research is being carried out to develop alternatives, like vein grafts, nerve conduit, and allografts. However, these alternatives have limited effectiveness in comparison to autogenous nerve grafting in the reconstruction of large nerve defects (>3cm), so it is usually used in bridging small defects (1–2cm) in small diameter nerves (Isaacs & Browne, 2014; Strauch et al., 1996; Ulkur, Yuksel, Acikel, Okar, & Celikoz, 2003).

Since the introduction of distraction osteogenesis principle by Ilizarov, bone lengthening or bone transport has been widely accepted for the treatment of various skeletal problems (Ilizarov, 1990). Several reports had shown that gradual lengthening of muscle and nerves occurred successfully during the bone lengthening without serious implications on the function (Abe et al., 2004; Ilizarov, 1989). This procedure introduced a new principle of distraction neurogenesis that might be a hopeful therapeutic tool for the reconstruction of nerve defects (Kroeber, Diao, Hida, & Liebenberg, 2001; Nishiura et al., 2006). Unfortunately, the literature lacks sufficient data of the pain study during the application of distraction neurogenesis as it is crucial to assess the pain as well as the functional nerve recovery.

The objective of our study was to evaluate the effectiveness of a newly designed mechanical prototype for the reconstruction of a 10-mm nerve defect of the sciatic nerve in rats compared to nerve autografting and also



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**Figure 1.** 3D view of the nerve-lengthening device design. It is composed of 3 parts: part 1 (in red) is fixed to the k-wire (in white), part 2 (in blue) can be rotated to move the suture filament attached through 2 holes, part 3 (in green) to handle the device during the movement of part 2.

to assess the pain during the period of distraction neurogenesis.

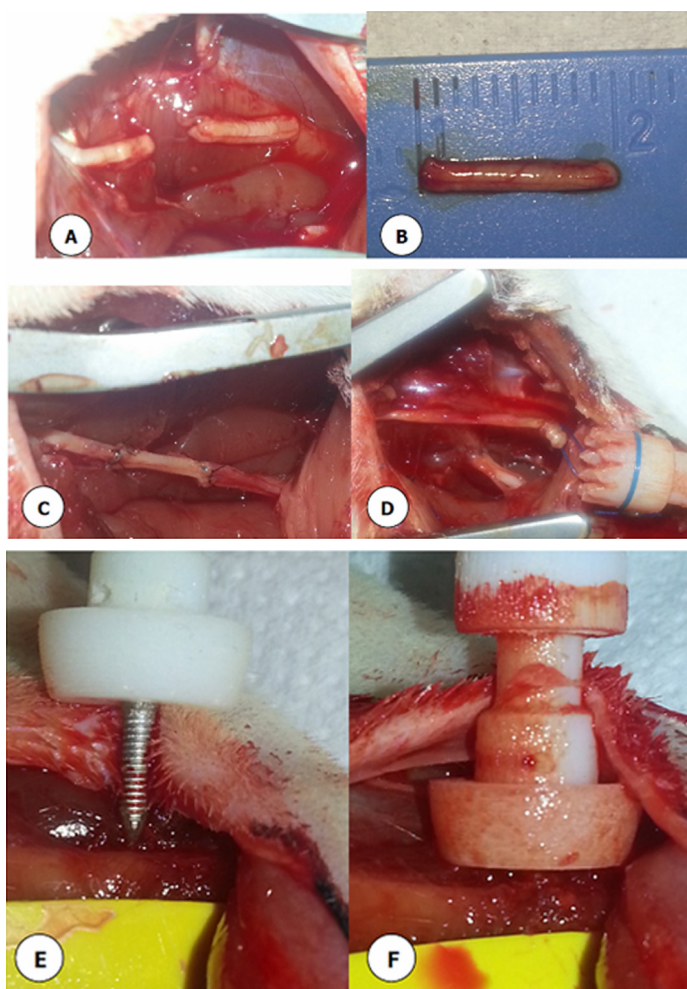
## 2. Methods

After approval of the Ethics Committee for animal experimentation of the University of Pavia, 14 Sprague Dawley rats were used with weight ranging from 225 to 250 mg. All international and institutional guidelines for the care and use of animals were followed. They were housed in 14 cages (one in each cage) with adjusted suitable environment as regards temperature, humidity, and ventilation with 12-h light and dark cycles. They also had free access to standard rat food and water. They were randomly assigned into 2 groups with 7 rats in each group; group A (Nerve Autografting group) in which a 10-mm segment of their sciatic nerve was resected and rotated 180 degrees. Then, primary end-to-end neurorrhaphy was performed in the reverse direction; group B (Nerve Lengthening group) in which the mechanical



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**Figure 2.** The final design of nerve-lengthening device.



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**Figure 3.** Surgical procedure. A) Sciatic nerve segment resection. B) 10-mm resected nerve segment. C) 180 degrees rotated nerve segment with primary end-to-end neurorrhaphy. D) Nerve lengthening of the proximal stump at the end of the experiment for secondary neurorrhaphy. E) The mechanical device was fixed to the femur with simple advancement of its self-tapping threaded tip against the bone. F) The shoulder of the motor unit flushed with the surface of the femur.

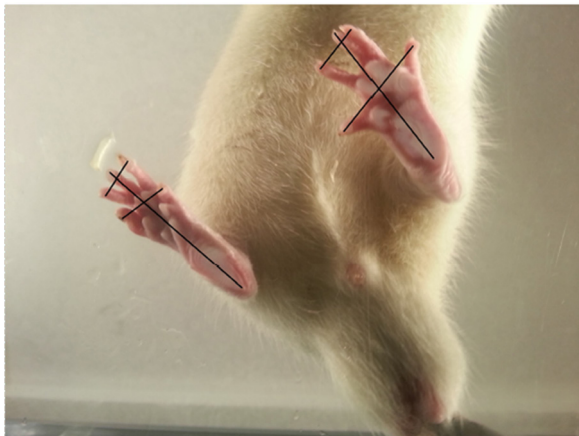
prototype, designed for applying longitudinal traction in terms of 1 mm/d upon the proximal stump of the sciatic nerve, was inserted after surgical resection of 10 mm of the sciatic nerve. Then, secondary end-to-end neurorrhaphy was performed after completing the nerve lengthening.

### 2.1. The mechanical device

The device was designed with SolidWorks software (Dassault Systèmes, France). It was made of 3 parts mounted on a 1.5 mm threaded K-wire (Mikai S.p.A, Italy). As demonstrated in Figure 1, it consists of 3 parts. Part 1 (in red) and part 2 (in blue) compose a pin-based positioning system to pull the surgical stitch filament

of 4/0 prolene sutured to the proximal nerve stump and pass through two small holes on part 2. The system is designed to pull 1 mm of filament at each movement from one pin to the next one on part 2. Part 3 (in green) is used to keep the device in position while rotating part 2 with respect to part 1.

The nerve-lengthening device has been manufactured using 3D printing technology. We used Objet30 Pro 3D printer (Objet-Stratasys, USA), a high resolution machine that works with photo-polymeric material in which the printing material is deployed on a building tray in layers of 28µm of thickness and cured by an UV lamp that hardens the material layer by layer. We used VeroWhite photo-polymeric material, which showed



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**Figure 4.** Measuring the distance from the first to the fifth toe, the toe spread (TS); and the distance from the second to the fourth toe, the intermediary toe spread (ITS) in both the normal and experimental hindlimb.

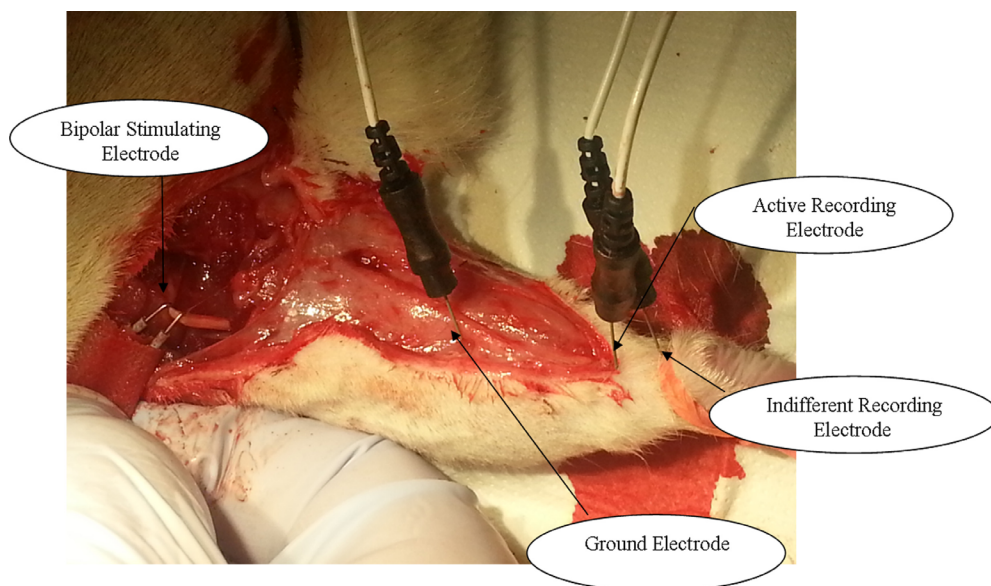
good mechanical properties for the specific application (Modulus of elasticity: 2000-3000 MPa and shore hardness (D): 83-86 Scale D). Actually, we must be ensured that the device pins withstand the tension of the wire and furthermore we had to guarantee a stable adherence of the plastic components to the k-wire.

A copy of the model can be prototyped in 45 minutes. The model was printed within a support resin that could be removed after the printing with the aid of a water jet machine and sodium hydroxide solution. Then, the prototyped parts were assembled on the k-wire of 30 mm of length and a diameter of 1.5 mm with a final threaded part of 4 mm. The 3D printed components were assem-

bled as in Figure 2, where parts 1 and 3 were fixed to the screw with a cyanoacrylate-based glue, while part 2 was free to move and kept in position by a spring.

## 2.2. Surgical procedure

Under general anesthesia induced with intramuscular injection of a combination of tiletamine hydrochloride, and zolazepam hydrochloride (Zolitel, Vibrac) with a dose of 10 mg/kg. The rat was stabilized in prone position on a woody tray with the use of elastic bands attached to forelimbs and hindlimbs and its back was shaved. Under aseptic condition (disinfection with povidone-iodine and alcohol 70% and draping with sterile towels), skin incision was made aseptically in the thigh along the posterior border of the femur. The overlying gluteal muscles were exposed and retracted with blunt dissection, and the sciatic nerve was exposed. Then, a 10-mm long nerve segment was resected (Figure 3A, 3B). The proximal level of nerve resection was at the level of greater trochanter of the femur while the distal level was just above the terminal division of the sciatic nerve. In group A, the resected 10-mm long nerve segment was rotated 180 degrees. Then, primary end-to-end neurorrhaphy was performed in the reverse direction with 9/0 nylon epineural sutures (Figure 3C). In group B, the mechanical device was inserted to be fixed with the distal part of the femur by simple advancement of its self-tapping threaded tip against the bone until the shoulder of the motor unit become flushed with the surface of the femur (Figure 3E, 3F). The traction sutures of 4/0 prolene were applied to the proximal nerve stump and then tied to the suture anchor hole of mechanical device.



**Figure 5.** Positions of the needle electrodes for EMG.

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The distal nerve stump was anchored to nearby soft tissues to prevent further retraction. Then, the wound was closed with a 2/0 silk suture. Stretching of the proximal nerve stump was started from the next day of the surgical intervention at a rate of 1 mm/d and repeated in the same time daily for 14 days under general anesthesia induced with inhalational diethyl ether (Sigma, Aldrich). We aimed to advance the proximal nerve stump approximately 3-5 mm past the distal stump to permit excision of the formed neuroma, so that the neurotomy would be performed without tension. No immobilization was applied postoperatively.

After carrying out lengthening, the nerve was exposed under the same previous surgical procedure, and observation was made for the presence of nerve stump overlapping (Figure 3D). Next, the nerve-lengthening device was removed. Both nerve stumps were refreshed with the excision of the neuroma, and direct end-to-end neurotomy was carried out with a 9/0 nylon epineurial sutures.

### 2.3. Outcome measures

Thirteen weeks after nerve resection, assessment of the functional recovery of the sciatic nerve using static sciatic index (SSI) was performed. Fourteen weeks after the nerve resection, assessment of the nerve regeneration with electrophysiological study and histological analysis were performed. Also, gastrocnemius wet weight was measured. For pain assessment in group B, Rat Grimace Scale (RGS) score was used.

#### 2.3.1. Rat grimace scale

A thorough study of pain using Rat Grimace Scale (RGS) score, was conducted throughout the whole period of nerve lengthening. This scale was shown to be a reliable and highly accurate measure of pain in laboratory rodents (Sotocinal et al., 2011). It consists of four facial action units (orbital tightening, nose/cheek bulge, ear position, and whisker change), each is scored on a 0-2 scale (0=not present, 1=moderately present, and 2=obviously present) (Sotocinal et al., 2011).

#### 2.3.2. Sciatic static index

Analysis of the footprint is the most commonly used non-invasive simple method for the evaluation of the functional recovery of the sciatic nerve in rats. Bervar (Bervar, 2000) introduced the sciatic static index (SSI), which is considered to be an effective method for the



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**Figure 6.** The Medelec Sapphire 1P EMG machine.

assessment of the functional recovery after sciatic nerve injury in rats.

It is based on recording the footprints, acquired while the rat is on a static position, and by this way it avoids the bias related to gait's velocity that may be encountered if the sciatic functional index (SFI) is calculated instead. Also, SSI has the advantage of improvement of footprints acquisition. Therefore, it is more accurate than the SFI (Baptista et al., 2007). Thirteen weeks after nerve resection, functional recovery of the sciatic nerve was assessed using video recording of the plantar aspect of the rat hindfeet when the rat rests on static position in a glass-bottomed box measuring 61×41×19 cm. The video camera was positioned underneath the transparent glass-bottomed of the box. Two 150 W light sources were placed on both sides of the glass box for better illumination. Under these conditions, a clearly visible acquired photo was chosen and the followings distances were measured (Figure 4): 1) distance from the first to the fifth toe, the toe spread (TS); and 2) distance from the second to the fourth toe, the intermediary toe spread (ITS) (Figure 4). All measurements were taken from both the experimental (E) and normal (N) side. Then, toe spread factor [TSF=(ETS-NTS)/NTS] and intermediary toe spread factor [ITF=(EIT-NIT)/NIT] were calculated and incorporated into Bervar sciatic static index formula (Bervar, 2000):

$$SSI=108.44 \text{ TSF}+31.85 \text{ ITF}-5.49$$

A total score of 0 is considered normal while score of -100 indicates total impairment, which would occur after complete transection of the sciatic nerve (Bervar, 2000). All video recordings were made under the same conditions as regards the time, light, and human activity patterns. A special attention was paid to remove any stress

that would affect the postural muscle tone of the rats. A tested rat was gently placed into the glass box, and then a minimum of 5 minutes was left before beginning the video recording to make the rat adapt well to the new environment. Only one rat in group B could not be evaluated due to partial absence of toes as a result of autotomy.

### 2.3.3. Electrophysiological studies

Fourteen weeks after the nerve resection, the sciatic nerve was exposed under general anesthesia in both groups using the same previous surgical technique with exposure and isolation of the sciatic nerve with gentle dissection. Electrical stimuli were applied to the sciatic nerve trunk at its proximal portion in a presection point at the sciatic foramen while latency and amplitude were recorded in the gastrocnemius muscle at the ipsilateral side. The stimuli used were direct current rectangular shocks (duration, 0.5 ms; frequency, 1 Hz). Voltage was set at the threshold required to obtain a supramaximal motor response. Bipolar stimulation electrode was used to deliver the electrical stimuli to the sciatic nerve with the cathode as the active electrode and 5 mm cathode to anode distance. The active recording “different” electrode was a needle electrode placed on the gastrocnemius muscle belly, and the reference “indifferent” recording needle electrode was inserted into the Achilles tendon (Figure 5).

Actually, the recorded muscle action potential is the difference between the action potential detected at these two electrodes due to the presence of frequent sources of action potential that we do not want to record. That is why we used the bipolar differential recording method for electromyography (EMG) with 2 recording electrodes. A differential amplifier was used to subtract the potential voltages at the two recording sites and amplify the difference in-between. The aim of such subtraction procedure was to significantly reduce the magnitude of other signals that come from elsewhere. The amplified signal was then fed into a computer. In order to ensure selective measurement of biopotentials, the rat body surface is usually grounded with a fourth (ground) needle electrode which was inserted in nonmuscular tissue between the stimulation and active recording electrode. The distance between the stimulation electrode and the active recording electrode was fixed at 38 mm. The needle electrodes were monopolar (Ambu® Neuroline Aghi Monopolari, Italy), constructed from stainless steel which was insulated, except at its tip. The average diameter of the needle was 0.36 mm and the length 15 mm.

In our experiment, we used the intramuscular needle recording electromyography rather than surface recording as it is extremely sensitive. In addition, the recorded surface EMG action potential activity of a muscle would be affected with the electrical activity that comes from other muscles (crosstalk) and that would make a false assessment of the muscle under investigation. Furthermore, the recorded surface EMG activity may even include electrical signals coming from the heart and the brain (Turker, 1993).

The electromyography studies were performed using the Medelec Sapphire 1P (Medelec Ltd, Surrey, England) (Figure 6), where the EMG electrical signals were displayed on an oscilloscope and the recorded electrical activities were printed, including latency (in milliseconds) and amplitude (in millivolts). Two repetitive recordings were registered in each electrophysiological study of the sciatic nerve on both the operated and contralateral normal side. Then, the best recording with high amplitude of muscle action potential was selected. Motor nerve conduction velocity (NCV) (m/s) was calculated by dividing the distance between the active stimulating and recording electrodes (in mm) by the latency over the same distance (in ms).

### 2.3.4. Gastrocnemius muscle weight

After performing the electrophysiological studies, the gastrocnemius muscle was harvested and weighted immediately on an electronic precision scale of both operated and normal side using E42-B model of PPS device (Gibertini elettronica, SRL, Italy).

### 2.3.5. Histological analysis

After measuring the gastrocnemius weight, the sciatic nerve of the operated hindlimb was harvested in both groups and fixed with 4% formaldehyde and kept in refrigerator at 4°C for 12 hours. Then 2 cut sections were made in each sampled sciatic nerve; one section in the intermediate segment, which included the autograft in group A and the regenerated lengthened nerve segment in group B; and the other section was made in the distal segment 3 mm distal to the nerve repair site. Then, washing of histological samples was made with phosphate buffered saline (0.1 M). Next, secondary fixation with osmium tetroxide (1%) in Sym-collidine buffer was performed for 1.5 hours, which provided excellent fixation and stable buffering. The pH was adjusted by varying the amount of hydrochloric acid, in a range of 7.3 to 7.7. Dehydration was made using ethanol alcohol starting with 25% and increasing progressively to 50%, 70%,

80%, and 90% every 5 minutes till reaching 95%, then kept overnight with alcohol 95%. Finally, alcohol 100% was used for 45 minutes. Propylene oxide was then used to remove the residual ethanol previously used for dehydration. The sample was first immersed in a mixture of equal volumes of ethanol and propylene oxide for 5 minutes, and then four times in pure oxide, 10 minutes each.

Finally, replacement of the transition solvent with epoxy resin "EPON 812" was performed, which is also capable of polymerization to form a rigid three dimensional structure with cross linking between molecular chains. Sections of 0.5  $\mu\text{m}$  thickness were prepared by a precision Ultracut microtome (Reichert-Jung, Germany) using sharply fine diamond blades. The sections were then stained with toluidine blue solution (0.5% toluidine blue, 1% sodium tetraborate dissolved in water). Examination of the stained sections was made using Axiophot microscope (Zeiss, Germany) connected with Nikon display screen. Photomicrographs were taken of a representative field at 63x magnification under oil immersion lens with the area frame equals 137x 102.75  $\mu\text{m}$ . We had used a semi-automated binary imaging analysis using Image J software for histological analysis (Schneider, Rasband, & Eliceiri, 2012). Fascicular and myelin area percentage, fiber density, fiber and axon diameter, myelin sheath thickness, myelin and axon surface area, G-Ratio, myelin/axon ratio, mean fiber surface area and fiber number per representative field were evaluated in each sample by an independent experienced pathologist in a blinded fashion.

## 2.4. Statistical analysis

All data are presented as mean $\pm$ SD. The Mann-Whitney U test was used to evaluate the differences between the 2 groups. The Wilcoxon signed-rank test was used to evaluate the difference of the amplitude, latency, and conduction velocity between the operated and normal contralateral hindlimb. For all comparisons, a value of  $P < 0.05$  is considered to be statistically significant.

## 3. Results

### 3.1. Rat grimace scale

The daily recording of the RGS during the fourteen days of nerve lengthening was carried out in group B and the mean is graphically presented in Figure 7.

### 3.2. Sciatic static index

The measured SSI of the rats of both groups had shown better functional recovery of the sciatic nerve in group B compared to group A (Table 1). A statistical analysis of the difference of the SSI between the 2 groups was conducted using Mann Whitney U test and was found to be statistically significant with P value of 0.0271.

### 3.3. Electrophysiological studies

The recorded latency and amplitude of the gastrocnemius muscle action potential in the operated and normal contralateral hindlimb were shown (Table 2). Although the mean amplitude in group B was better than that recorded in group A, statistical analysis of the difference using Mann-Whitney U test was found to be statistically insignificant with P value of 0.174. However, a statistical significant difference was observed in comparing the amplitude of the operated hindlimb of group A and group B with that of the normal contralateral hindlimb using Wilcoxon Signed-Rank test with P values of 0.018 and 0.028, respectively.

Moreover, the latency in group B was also better than that recorded in group A and statistical analysis of the difference using Mann-Whitney U test was found to be statistically significant with P value of 0.035. A statistical significant difference was observed in comparing the latency of the operated hindlimb in group A with that of the normal contralateral hindlimb using Wilcoxon Signed-Rank test ( $P=0.018$ ), but no statistically significant difference was observed in comparing the latency of the operated hindlimb in group B with that of the normal contralateral hind limb using Wilcoxon Signed-Rank test ( $P=0.786$ ).

As regards the nerve conduction velocity (Table 3), better results were observed in group B compared to group A. Statistical analysis of the difference of the NCV between the two groups was performed using Mann-Whitney U test and it was found to be statistically significant ( $P=0.038$ ). A statistical significant difference was observed in comparing the NCV of the operated hindlimb in group A with that of the normal contralateral hindlimb using Wilcoxon Signed-Rank test with P value of 0.018, meanwhile no statistically significant difference was observed in comparing the NCV of the operated hindlimb in group B with that of the normal contralateral hindlimb using Wilcoxon Signed-Rank test ( $P=0.983$ ).

**Table 1.** Descriptive statistics of the SSI scores in both groups.

	Minimum	Maximum	Mean	Std. Deviation
Nerve Autograft group (A)	-42.11	-86.49	-64.4957	14.47546
Nerve lengthening group (B)	-24.36	-63.74	-41.5900	12.67766

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### 3.4. Gastrocnemius muscle weight

Although the average gastrocnemius muscle weight of group B was more than that of group A as demonstrated in Table 4, no statistically significant difference was observed using Wilcoxon Signed-Rank test (P value=0.225).

myelin/axon ratio on histological analysis of the distal nerve segment (Figure 8).

### 4. Discussion

Reconstruction of nerve defects is considered a challenging problem in reconstructive microsurgery so an

**Table 2.** Descriptive statistics of the recorded latency and amplitude in the operated and normal contralateral sciatic nerve in both experimental groups.

	Minimum	Maximum	Mean	Std. Deviation
Latency in autograft group (A)	1.75	3.35	2.5643	0.54061
Amplitude in autograft group (A)	4.83	16.90	11.6257	3.98038
Latency in contralateral limb of autograft group (A)	1.20	1.95	1.6000	0.24495
Amplitude in contralateral limb of autograft group (A)	17.80	67.60	35.8286	17.94034
Latency in nerve lengthening group (B)	1.55	2.25	1.8667	0.27689
Amplitude in nerve lengthening group (B)	8.25	30.80	16.8750	7.59748
Latency in contralateral limb of nerve lengthening group (B)	1.30	2.30	1.7714	0.31867
Amplitude in contralateral limb of nerve lengthening group (B)	31.10	40.00	33.4714	3.30541

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### 3.5. Histological analysis

Histological parameters of the intermediate and distal nerve segments were reported in both groups and compared with Mann-Whitney U test to detect any statistically significant difference (Table 5,6). Statistically significant difference was detected with regard to the fascicular area percentage and fiber density on histological analysis of the intermediate nerve segment meanwhile statistically significant difference was detected as regards the mean myelin sheath thickness, G-ratio, and

intensive research is carried out to develop effective alternatives to the standard nerve autografts. These alternatives comprise allograft, artificial nerve conduit, vein grafting, stem cell therapy, and neurotrophic factors (Chalfoun, Wirth, & Evans, 2006). However, none of the emerging methods had achieved better neurological recovery in comparison to the standard autografting particularly in cases of reconstruction of large gaps (Isaacs & Browne, 2014; Lundborg et al., 1982; Rich, Alexander, Pryor, & Hollowell, 1989). Lengthening of the periph-

**Table 3.** Descriptive statistics of the conduction velocity in both experimental groups.

	Minimum	Maximum	Mean	Std. Deviation
Conduction velocity in the operated hindlimb of autograft group (A)	11.34	21.71	15.4589	3.61602
Conduction velocity in the contralateral hindlimb of the autograft group (A)	19.49	31.67	24.2721	4.03371
Conduction velocity in the operated hindlimb of nerve lengthening group (B)	16.89	24.52	20.7343	3.06584
Conduction velocity in the contralateral hindlimb of nerve lengthening group (B)	16.52	29.23	22.0629	4.05385

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**Table 4.** Descriptive statistics of gastrocnemius muscle weight measurement in both experimental groups.

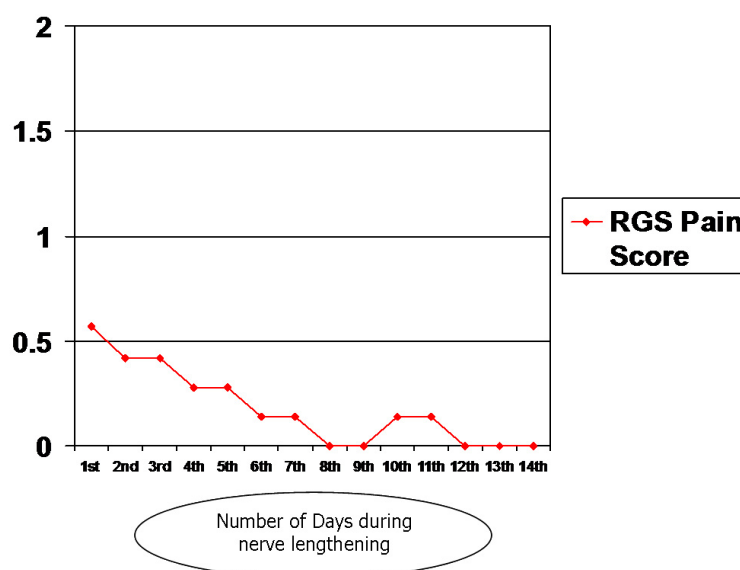
	Minimum	Maximum	Mean	Std. Deviation
Gastrocnemius weight in operated hindlimb of autograft group (A)	0.82	1.89	1.4549	0.35179
Gastrocnemius weight in contralateral hindlimb of autograft group (A)	2.26	3.58	2.9696	0.47599
Gastrocnemius weight in operated hindlimb of nerve Lengthening group (B)	0.65	3.27	1.8973	0.88324
Gastrocnemius weight in contralateral hindlimb of nerve Lengthening group (B)	2.39	3.51	2.8084	0.44044

eral nerves is a promising hope as a novel therapeutic tool in which the regenerating axons will cross through a single anastomotic site instead of two (with the standard autografting) besides avoiding the discrepancy between the nerve autograft and cut nerve stumps which in turn would theoretically improve the rate of nerve regeneration (Hara et al., 2012). Nerve axons respond to applied tension as a viscoelastic solid and axonal growth was enhanced in response to experimentally applied mechanical loading (Bray, 1984; Dennerll, Lamoureux, Buxbaum, & Heidemann, 1989). In addition, peripheral nerves can be safely elongated at a rate 1mm/d without histological damage (Abe et al., 2004).

Tissue expanders had been previously used to exert mechanical loading on either of the proximal or distal nerve stump to accomplish nerve elongation. They had achieved satisfactory functional results in comparison to nerve autograft (Baoguo, Shibata, Matsuzaki, & Takahashi, 2004; Matsuzaki, Shibata, Jiang, & Takahashi, 2004). However, tissue expanders also exert compression loads on the regenerated nerve that could interfere

with the functional nerve recovery with poor outcome in addition to the spherical shape of the expander which leads to unequal lengthening rate and lack of uniformity of the lengthened nerve segment (Sharula et al., 2010; Vaz, Brown, & Shah, 2014). Recently, an external fixator was developed by the University of Tsukuba, in Japan and was used for the reconstruction of nerve defects via nerve lengthening of both the proximal and distal stumps in different experimental species (Hara et al., 2012; Nishiura, Hara, Yoshii, & Ochiai, 2008; Sharula et al., 2010).

However, the mechanical device is bulky that may preclude its future clinical application on human studies. Also, despite the theoretical advantage of applying mechanical loading on both nerve stumps to reduce the period of nerve lengthening, regenerating axons of the proximal stump will travel toward their target for a longer distance through a mechanically disturbed environment if mechanical loading is applied to the distal stump (Vaz et al., 2014), because mechanical loading of the proximal stump of transected nerve is better tolerated in



**Figure 7.** Graphical presentation of the mean RGS pain score of the rats of group (B) during the period of nerve lengthening.

**Table 5.** Comparative statistical analysis of the histological parameters of the intermediate nerve segment.

	Group A	Group B	P Value
Fascicular Area%	42.04±7.03	33.28±2.77	0.03846*
Myelin Area%	32.17±4.8	27.03±2.82	0.07346
Fiber Density/mm <sup>2</sup>	32.172±6.516	25.223±3.128	0.01828*
Mean Fiber Diameter (μm)	3.840±0.242	3.9023±0.225	0.61708
Mean Axon Diameter (μm)	2.4686±0.636	2.6294±0.191	0.83366
Mean Myelin Sheath Thickness (μm)	1.4228±0.532	1.2720±0.182	0.9442
G-Ratio	0.63±0.15	0.66±0.03	0.71884
Average Myelin Surface Area (μm <sup>2</sup> )	10.3546±1.592	11.042±0.993	0.52218
Average Axon Surface Area (μm <sup>2</sup> )	5.333±2.044	5.6673±0.878	0.83366
Ratio Myelin/Axon	2.81±2.73	1.98±0.29	0.61708
Mean Fiber Surface Area (μm <sup>2</sup> )	13.1827±1.692	12.7601±2.117	0.89656
Fiber Number/Representative field	462±92.77	356.17±44.17	0.01828*

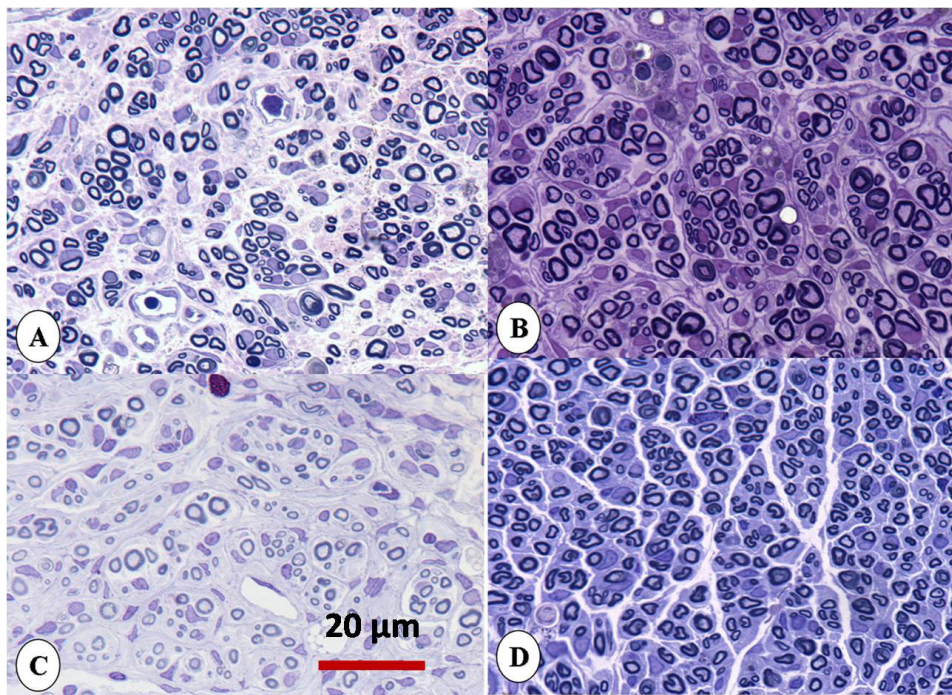
\* Denotes a statistically significant difference when P value <0.05

NEUR SCIENCE

terms of functional recovery than distal stump elongation (Skoulis, Lovice, von Fricken, & Terzis, 1995).

In our study, we developed a novel mechanical device using 3D printing technology that was designed to ap-

ply mechanical loading on the proximal stump of the resected nerve segment at a rate of 1mm/d. Our study had shown that the application of mechanical loads on the proximal nerve stump with the new device was success-



NEUR SCIENCE

**Figure 8.** Light microscopic photomicrographs of the histological specimens of the sciatic nerve magnified at 63x A) Nerve section at the intermediate nerve segment of the autograft in group A. B) Nerve section at the area of lengthened regenerated nerve segment in group B. C) Nerve section of the distal nerve segment at 3 mm distal to the repair site in group A. D) Nerve section of the distal nerve segment at 3 mm distal to the repair site in group B.

**Table 6.** Comparative statistical analysis of the histological parameters of the distal nerve sample.

	Group A	Group B	P Value
Fascicular Area%	24.5003±6.81	23.2824±7.288	0.83366
Myelin Area%	18.3650±4.7	19.7311±6.708	0.42952
Fiber Density/mm <sup>2</sup>	21.26967±5,861	19,24214±5,916	0.61708
Mean Fiber Diameter (µm)	3.638833±0.274	3.737257±0.458	0.83366
Mean Axon Diameter (µm)	2.6902±0.183	2.494257±0.286	0.35238
Mean Myelin Sheath Thickness (µm)	0.948633±0.174	1.332833±0.275	0.02034*
G-Ratio	0.7283±0.03	0.6657±0.07	0.04363*
Average Myelin Surface Area (µm <sup>2</sup> )	8.91067±0.761	10.571±2.939	0.35238
Average Axon Surface Area (µm <sup>2</sup> )	6.026±0.883	5.33671±0.994	0.35238
Ratio Myelin/Axon	1.4965±0.172	2.0163±0.585	0.01828*
Mean Fiber Surface Area (µm <sup>2</sup> )	11.6105±1.838	12.2834±3.217	0.71884
Fiber Number/Representative field	300.67±82.91	271.71±83.54	0.61708

\* Denotes a statistically significant difference when P value <0.05

NEURSCIENCE

ful for the reconstruction of a 10-mm segmental nerve defect in rats. We reported a significant better functional recovery rate (using the SSI) in the nerve lengthening group in comparison to autografting group 13 weeks after nerve resection. Furthermore, a statistically significant higher nerve conduction velocity was measured in the nerve lengthening group 14 weeks after nerve resection. On histological analysis of the distal nerve section at 3 mm distal to the nerve repair site, significant myelin sheath thickness was detected in the nerve lengthening group.

The better results reported in rats treated with nerve lengthening can be attributed to the maintenance of vascularity of the elongated proximal nerve segment, which enhances the nerve regeneration with better myelination of the regenerating axons instead of the avascular nature of the nerve autograft. Despite the theoretical advantage of a single neurorrhaphy site in case of nerve lengthening (that would enhance the number of the crossing regenerating axons), no significant difference was detected with regard to the nerve fiber density of the distal nerve segment.

Assessment of pain during nerve lengthening demonstrated stepwise decrease of pain starting from the first day postoperatively (mean score=0.57) to the eighth day (mean score=0.14) using RGS. An episode of pain was detected on the 10<sup>th</sup> and 11<sup>th</sup> days (mean score=0.14). The maximum severity of pain (0.57) reported on the first day was considered mild pain. The potential drawbacks of possible therapeutic application for distraction

neurogenesis in the reconstruction of nerve defects in future human studies, would be the necessity of operating the patients twice and also the device-related problems, like mechanical failure and infection.

We believe that distraction neurogenesis with the new experimental device is a reliable therapeutic method for the reconstruction of nerve defects that results in a better functional recovery rate superior to that obtained with the standard nerve autografting.

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