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*Article*

# A Single Postoperative Application of Defocused Low-Energy ESWT Does Not Significantly Enhance Regeneration of the Rat Median Nerve Following Reconstruction with Autologous Nerve Grafts or Muscle-In-Vein Conduits

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**Abstract:** Investigations reporting positive effects of Extracorporeal Shock Wave Therapy (ESWT) on nerve regeneration are limited to the rat sciatic nerve model. The effects of ESWT on muscle-in-vein conduits (MVCs) have also not been investigated yet. This study aimed to evaluate the effects of ESWT after repair of the rat median nerve with either autografts (ANGs) or MVCs. In male Lewis rats, a 7-mm segment of the right median nerve was reconstructed either with an ANG or MVC. For each reconstructive technique, one group of animals received one application of ESWT while the other rats served as controls. Animals were observed for 12 weeks and nerve regeneration was assessed via computerized gait analysis, the grasping test, electrophysiological evaluations and histological quantification of axons, blood vessels and lymphatic vasculature. Here we provide for the first time a comprehensive analysis of ESWT effects on nerve regeneration in a rat model of median nerve injury. Furthermore, this study is among the first reporting the quantification of lymphatic vessels following peripheral nerve injury and reconstruction in vivo. While we found no significant direct positive effects of ESWT on peripheral nerve regeneration, results following nerve repair with MVCs were significantly inferior to those after ANG repair.

**Keywords:** nerve repair; median nerve; rat; autologous nerve graft; muscle-in-vein conduit; extracorporeal shock wave therapy; grasping test; gait analysis; CatWalk, nerve regeneration;

## 1. Introduction

Peripheral nerve injuries implicate severe physical [1-4] and psychosocial impairments [5,6] for the affected patients. Depending on the degree of nerve injury, surgical treatment may be necessary to restore the affected nerve's function, but if the nerve continuity has been lost entirely, e.g. neurotmesis, a surgical intervention is obligatory [7,8].

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Reconstruction of segmental nerve injuries poses another clinical problem, given the influence of graft length and scarring at the coaptation sites on nerve regeneration [9-11]. While nerve autografts (ANGs) are considered the gold-standard treatment option for segmental nerve injuries, their use is restricted by their limited availability throughout the body, donor site morbidity resulting from harvesting them and specific requirements in regard to graft diameter and vascularization [12,13]. Therefore, non-invasive treatment options to enhance axonal regeneration, target organ reinnervation and functional recovery [14] are sought for by the scientific community [15,16]. Among these approaches, extracorporeal shock wave therapy (ESWT) was reported by several authors to exert significant proregenerative effects on lesioned peripheral nerves [17-20]. Shock waves, sonic pulses with high energy impact, exert their effects on target tissues by biochemical changes induced by mechanotransduction. Among these effects, improved vascularization via the activation of nitric oxide synthase (NOS), an increased expression of growth factors like activating transcription factor 3 (ATF-3) and growth-associated phosphoprotein 43 (GAP-43), local anti-inflammatory effects and influencing of target cells too are thought to be the main drivers for improved tissue and nerve regeneration following ESWT [21-23]. Schwann cells proliferation and phenotype is also directly influenced by ESWT, enhancing peripheral nerve regeneration through activation of these glial cells [18,24-26]. Application of ESWT to improve nerve regeneration was first described by Hausner et al. in a rat model of sciatic nerve autograft repair [27]. The sciatic nerve injury model, especially in case of neurotmesis injuries, has several drawbacks mostly related to compromised animal welfare due to the onset of neuropathic pain, joint contractures and automutilation [28,29]. As the sciatic nerve supplies innervation both to flexor and extensor muscles of the hind paw, misdirection of axons can easily occur following neurotmesis injuries in which by definition the fascicular structure is lost. Misdirected axons in turn will either innervate the wrong target organ, e.g. an efferent axon regrowing into the skin or a muscle acting antagonistic to the axon's original target organ. In consequence, the subtle balance of agonistic and antagonistic muscles will be lost, severely impairing functional recovery following nerve injury [30,31]. In conclusion the overall potential of functional recovery is limited in rats with sciatic nerve injury in addition to difficulties evaluating it due to the aforementioned reasons [32,33]. The median nerve model of the rat which was first described by Bertelli [34,35] about 30 years ago offers a valid alternative given that the occurrence of limb contractures, severe neuropathic pain and automutilation is far less frequently observed in comparison to sciatic nerve injuries [36]. Furthermore, functional recovery can be evaluated by means of the grasping test [37], staircase test [38] and computerized gait analysis [39] in addition to electrophysiological testing and histological analysis of the regenerating nerve [40,41]. Besides these considerations regarding the choice of an appropriate animal model, the use of ESWT in preclinical studies of segmental nerve injuries remains limited to ANGs and the effects of ESWT on non-nervous grafts have not been reported yet. Muscle-in-vein conduits (MVCs) which were first described by Bertelli in the 1990s are an alternative to reconstruct segmental nerve injuries and promising results have been published following their clinical application [42-44]. We have recently reviewed the results of nerve repair by means of MVCs both in preclinical and clinical research [45]. This review's main findings were significant differences in regard to functional recovery between animal studies and human studies utilizing MVCs to reconstruct segmental lesions of peripheral nerves. We hypothesized different experimental settings and profound inter-species differences in neurobiology to be the main reasons for this observation. In conclusion we advised for further studies to be conducted investigating the results of nerve reconstruction by means of MVCs and potential approaches to tackle the likely biological hurdles impeding nerve regeneration through them. Given this lack of studies investigating potential pro-regenerative effects of ESWT in a murine model of forelimb nerve injury on the one hand and the interplay of ESWT and nerve reconstruction by means of MVCs, we designed an in vivo study addressing both these research questions at hand. It was the aim of our study to test the hypothesis

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that a single postoperative application of ESWT immediately can enhance peripheral nerve regeneration following reconstruction of the rat median nerve with either ANGs or MVCs.

*Animals and Surgery*

The experimental protocol was approved beforehand on July 23rd, 2019, by the Animal Protocol Review Board of the City Government of Vienna (Magistrate's office number 58, Project identification code: MA58-421715-2019-16). All procedures were carried out in full accord with the Helsinki Declaration on Animal Rights and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Fifty-six male Lewis rats (Janvier Labs, Le Genest-Saint-Isle, France), weighing 280–350 g, were kept in groups of two or three in appropriate cages according to internal standard operating procedures. The animals had access to food and water ad libitum. After the rats were allowed to get accustomed to their new surrounding for 7 days prior to any experimental handling and after completing a 7-day training period on the CW device, they were randomly assigned to the following groups: median nerve reconstruction with autologous nerve grafts (ANGs) (total *n*=29), median nerve reconstruction with MVCs (total *n*=27). Both groups were each further subdivided into a group of animals which received ESWT; ANG+ESWT (*n*=15), MVC+ESWT (*n*=11) and a group of animals which received no additional treatment; ANG (*n*=14), MVC (*n*=16), serving as controls. Therefore, a total of four groups of animals were investigated in this study. After random group allocation, the rats underwent bilateral surgery of the median nerve under an operation microscope (Leica M651, Leica Microsystems, Vienna, Austria). A 7-mm segment of the left and right nerve was removed by performing a transection about 1.5 mm proximal to the position where it is crossed over by the brachial artery and vein and another transection 7mm proximal to the first one. On the right side, the gap was bridged with either the original nerve segment in reverse fashion as a homotopic ANG or an MVC. The MVCs were prepared by introducing several muscle fibers of the left gracilis muscle into a segment of the epigastric vein as described elsewhere [38]. ANGs and MVCs were coaptated with the proximal and distal stump of the median nerve with two sutures per coaptation site (Ethilon, 10-0, Ethicon-Johnson & Johnson, Brussels, Belgium). On the left side, the nerve defect remained unreconstructed to serve as an internal control group. To prevent spontaneous regeneration, the distal nerve stump was sutured into the short head of the biceps muscle. The post-operative observation period lasted 12 weeks. At the end of the postoperative observation periods the rats were sacrificed in deep anesthesia induced as described above via intracardial puncture and administration of an overdose of sodium thiopental.

*Application of ESWT*

Following median nerve reconstruction with an ANG or MVC, rats in both the ANG+ESWT group (*n*=15) and the MVC+ESWT group (*n*=11) received 300 impulses (3 Hz, 0.1 mJ/mm$^{2)}$ of ESWT (OP 155 connected to Orthogold 100, MTS Medical, Konstanz, Germany) while still in deep anesthesia to prevent movement-induced artifacts. Focused application of ESWT was facilitated by use of a 3D-printed customized device in which the right forelimb was introduced and fixed using a noose made from elastomer (Supplementary Figure 1). The area between the applicator and the rat's right forelimb was filled with ultrasonic transmission gel to guarantee adequate and reproducible transmission of impulses.

**Functional Analysis**

*Reflex-based grasping*

Motor function of the superficial finger flexor muscle (FDS) and deep finger flexor muscle (FDP) was evaluated weekly by means of the grasping test as originally described

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by Bertelli [46] and modified by us [39] and other authors [38]. As the FDS and FDP in rats are predominantly innervated by the median nerve, the ability to flex the toes of the forelimbs is mediated by this nerve [39,41,46]. We recorded three trials per week and only those trials were deemed valid in which no flexion of the elbow (biceps muscle) or wrist (flexor carpi ulnaris muscle and flexor carpi radialis muscle) were evident. Return of grasping ability in general was grading as described by Stößel et al.; **1/3:** no observable toe flexion, **2/3:** toe flexion without measurable strength when forced to pull the bar, **3/3:** toe flexion with measurable strength when forced to pull the bar [38].

*CatWalk XT gait analysis*

To evaluate changes in gait behavior, computerized gait analysis was performed biweekly using the CatWalk XT gait analysis system as described elsewhere [39,47,48]. The device consists of a walkway with a glass floor which is illuminated by green and red ceiling light sources. While the green light source illuminates a crossing rat's or mice's paws, the red ceiling light's provides contrast for the animal's body contour. The acquired images are then recorded by a fully automated camera mounted underneath the glass plate and processed by the system's software. During the course of a seven-day training period, the animals were habituated to cross the walkway with at a speed between 50 and 100 cm/s [39]. Following completion of each data acquisition session, the animals were rewarded with 1–2 pellets of cereal. We assessed the following parameters: Print Area Ratio of the right front paw (RF) and right hind paw (RH) (%) Print Length Ratio RF/RH (%), Print Width Ratio RF/RH (%), Swing Speed Ratio (RF/RH) (%), Swing Time Ratio RF/RH (%), Duty Cycle Ratio RF/RH (%), Stand Index Ratio RF/RH (%), Front Paw Base of Support (FP BoS) (%) and Ulnar Abduction of the RF [39].

*Electrophysiological Analysis*

At the end of the twelve-week observation period rats underwent electrophysiological evaluations with a Neuromax EMG device (Natus, WI, United States) as described elsewhere [39]. Briefly, the right median nerve was gently freed from its surrounding tissue. The recording electrode was placed inside the flexor digitorum superficialis muscle while the reference electrode was placed in the ipsilateral paw. The grounding electrode was subcutaneously inserted in the right hind limb. Using a micromanipulator, a bipolar stimulation electrode was positioned 2-3mm proximal to the proximal coaptation site. Latency and Compound muscle action potential (CMAP) of the flexor digitorum superficialis muscle were measured using supramaximal stimulation. Measurements were normalized with the animal's core temperature which was assessed rectally.

*Wet muscle weight*

Following sacrification of the animal. both the right and left flexor digitorum superficiales muscle were harvested and weighted. The weight of the right FDS muscle was normalized both to the weight of the contralateral, chronically denervated muscle as well as to the animal's body weight.

*Histological Analysis*

To obtain the correct position as well as distal and proximal orientation of the nerves, they were pinned with minutius needles on small styropor stubs. For histochemical and immunohistochemical stainings, the nerves were fixed in 4% buffered formalin for 24 hours at room temperature and afterwards rinsed in tap water for 1 hour. Dehydration with an uprising ethanol series was performed, beginning with 50% EtOH for 1 hour, followed by 70% EtOH. Then, the samples were transferred to a vacuum infiltration processor (Sakura, TissueTek ® VIP) and after further dehydration of the samples, infiltrated with paraffin via the intermedium of xylene. Cutting the nerve samples in 4 µm thin cross-sections was performed with an Microm HM355S (ThermoScientific). After drying the

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sections overnight in a 37°C oven, the slides were deparaffinized and rehydrated for staining with different methods. Nuclei are stained in grey using Weigert's Iron Haematoxylin. After staining, the sections are dehydrated and permanently embedded with Shandon Consul-Mount (Thermo Scientific). Starting immunohistochemical stainings, the sections were first pre-treated with different antigen retrieval protocols. For S100 (Agilent, Z0311) the sections were incubated with Pepsin (Sigma) for 10min at 37°C in a humidified chamber. The sections for Podoplanin (ReliaTech, 104-M40) staining were steamed in a pH 6 sodium citrate buffer (0.1 M) for 20min, for CD31 (Thermo, PA5-16301) with EDTA buffer (0.1 M) at pH 9. After the antigen retrieval, the sections were blocked using Bloxall® (VectorLabs) for 10 min. Then, the primary antibodies were applied for 1 hour at room temperature (S100 1:1600, Podoplanin 1:2000, CD31 1:50) followed by incubation of secondary antibodies for 30 min at room temperature using an HRP conjugated anti-mouse system (ImmunoLogic, VWRKDPVM110HRP) for S100 and Podoplanin. For CD31 an anti-rabbit HRP conjugated antibody was used (ImmunoLogic, VWRKDPVR110HRP). The detection of the staining was performed with ImmPACTTM NovaREDTM (VectorLabs). Then the sections were counterstained with Haematoxylin and after dehydration permanently embedded with Shandon Consul-Mount (Thermo Scientific).

*Automated quantification of axons, lymphatic and blood vessels*

We employed automated deep learning based image analysis to quantify the axon and lymphatic vessel count in whole-slide scans of histological cross-sections. The IKOSA platform (KML Vision) was adopted to train two state-of-the-art deep neural network models in a supervised fashion. To quantify the lymphatic vessels, we applied our previously trained model to our image data as described elsewhere [49].

A second model was trained to segment axons in the digital images. To improve the ground truth data annotation quality, regions of interest (ROI) were defined to restrict the area where axons were marked using the annotation tools provided by IKOSA. A set of 54 whole-slide scans containing 149 ROI was randomly split into training (48 images, 116 ROI) and validation (6 images, 33 ROI) data. The model training converged after 4 hours 43 minutes on GPU infrastructure. The validation performance at the axon instance count is 95.4% recall and 94.2% precision. See **Supplementary Table 1** and **Supplementary Figure 2** for more details on the dataset and validation statistics.

The blood vessel count in the cross-sections are reported as manual counts.

*Statistical Analysis*

All statistical analyses were performed using IBM SPSS Version 26 (International Business Machines Corporation, Armonk, NY, USA. For each parameter normal distribution was tested by means of the Kolmogorov–Smirnov–Test. Homogeneity of variances was tested with Levene's test. In case both criteria were met, data were compared with parametric tests, e.g. one-way analysis of variance (ANOVA). This was followed by the Tukey post-hoc-test. Otherwise, non-parametric comparisons, e.g. the Kruskal-Wallis test, followed by the Dunn-Bonferroni post-hoc-test were used for comparison for more than 2 groups. Sub-analysis of groups (ANG VS MVI; ANG VS ANG+ESWT, MVI VS MVI+ESWT) was performed with the Mann-Whitney-U-Test in case of non-normally distributed data, otherwise student's t-test was used. Repeated measures of the same sample were compared with the non-parametric Friedman test. P-values $< 0.05$ were considered statistically significant. All values are expressed as mean ± 1 standard error of the mean (SEM).

## 3. Results

*Functional analysis*

Reflex based grasping

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By using the grasping test we aimed to evaluate the return of general grasping ability in general and grasping strength in particular as both depend on reinnervation of the flexor digitorum superficialis muscle (FDS) one of the median nerve's target organs.

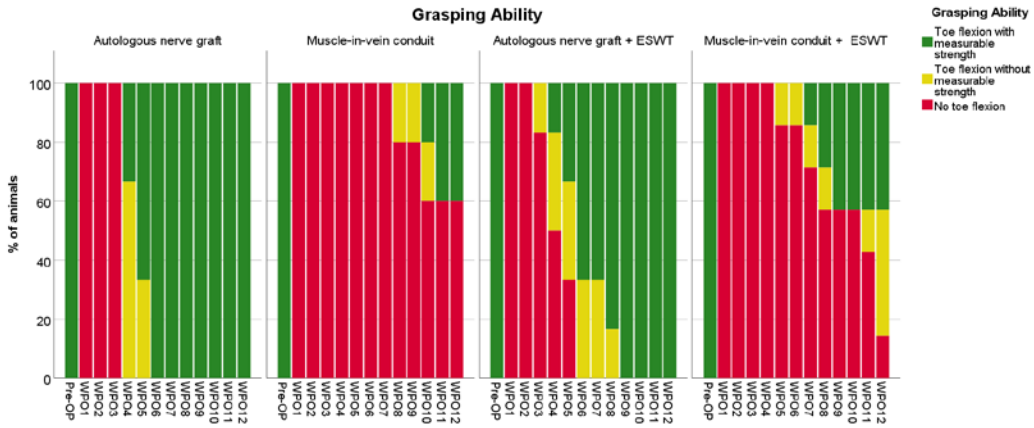


**Figure 1.** Recovery of grasping ability following reconstruction of the right median nerve during the postoperative 12-week observation period. Autologous nerve graft (n=3); Muscle-in-vein conduit (n=5); **Autologous nerve graft + ESWT:** Autologous nerve graft with postoperative extracorporeal shockwave therapy (n=6); **Muscle-in-vein conduit + ESWT**: Muscle-in-vein conduit with postoperative extracorporeal shockwave therapy (n=7). **WPO:** postoperative week.

**Table 1.** Summary of functional recovery as assessed by the grasping ability (**1/3:** No toe flexion; **2/3:** Toe flexion without measurable strength; **3/3:** Toe flexion with measurable strength) during the 12-week postoperative observation period. Statistical differences were tested with the non-parametric Kruskal-Wallis test and the Dunn-Bonferroni post-hoc-test. **ANG:** Autologous nerve graft (n=3); **MVC:** Muscle-in-vein conduit (n=5), **ANG + ESWT:** Autologous nerve graft with postoperative extracorporeal shockwave therapy (n=6), **MVC + ESWT:** Muscle-in-vein conduit with postoperative extracorporeal shockwave therapy (n=7). *: $p < 0.05$ vs ANG; ●: $p < 0.05$ vs. MVC; ▲: $p < 0.05$ vs ANG + ESWT; #: $p < 0.05$ vs. MVC + ESWT. **WPO:** postoperative week.

| WPO1 | | | |
|---|---|---|---|
| Ability | 1/3 | 2/3 | 3/3 |
| ANG | 3/3 (100%) | 0/3 (0%) | 0/3 (0%) |
| MVC | 5/5 (100%) | 0/5 (0%) | 0/5 (0%) |
| ANG + ESWT | 6/6 (100%) | 0/6 (0%) | 0/6 (0%) |
| MVC + ESWT | 7/7 (100%) | 0/7 (0%) | 0/7 (0%) |

| WPO2 | | | |
|---|---|---|---|
| Ability | 1/3 | 2/3 | 3/3 |
| ANG | 3/3 (100%) | 0/3 (0%) | 0/3 (0%) |
| MVC | 5/5 (100%) | 0/5 (0%) | 0/5 (0%) |
| ANG + ESWT | 6/6 (100%) | 0/6 (0%) | 0/6 (0%) |
| MVC + ESWT | 7/7 (100%) | 0/7 (0%) | 0/7 (0%) |

| WPO3 | | | |
|---|---|---|---|
| Ability | 1/3 | 2/3 | 3/3 |
| ANG | 3/3 (100%) | 0/3 (0%) | 0/3 (0%) |
| MVC | 5/5 (100%) | 0/5 (0%) | 0/5 (0%) |
| ANG + ESWT | 5/6 (83,3%) | 1/6 (16,7%) | 0/6 (0%) |
| MVC + ESWT | 7/7 (100%) | 0/7 (0%) | 0/7 (0%) |

| WPO4 | | | |
|---|---|---|---|
| Ability | 1/3 | 2/3 | 3/3 |
| ANG ● # | 0/3 (0%) | 2/3 (66%) | 1/3 (33%) |
| MVC * | 5/5 (100%) | 0/5 (0%) | 0/5 (0%) |
| ANG + ESWT | 3/6 (50%) | 2/6 (33,3%) | 1/6 (16,7%) |
| MVC + ESWT * | 7/7 (100%) | 0/7 (0%) | 0/7 (0%) |

| WPO5 | | | |
|---|---|---|---|
| Ability | 1/3 | 2/3 | 3/3 |
| ANG ● # | 0/3 (0%) | 1/3 (33,3%) | 2/3 (66,7%) |
| MVC * | 5/5 (100%) | 0/5 (0%) | 0/5 (0%) |
| ANG + ESWT | 2/6 (33,3%) | 2/6 (33,3%) | 2/6 (33,3%) |
| MVC + ESWT * | 6/7 (85,7%) | 1/7 (14,3%) | 0/7 (0%) |

| WPO6 | | | |
|---|---|---|---|
| Ability | 1/3 | 2/3 | 3/3 |
| ANG ● # | 0/3 (0%) | 0/3 (0%) | 3/3 (100%) |
| MVC * ▲ | 5/5 (100%) | 0/5 (0%) | 0/5 (0%) |
| ANG + ESWT ● # | 0/6 (0%) | 2/6 (33,3%) | 4/6 (66,7%) |
| MVC + ESWT * ▲ | 6/7 (85,7%) | 1/7 (14,3%) | 0/7 (0%) |

| WPO7 | | | |
|---|---|---|---|
| Ability | 1/3 | 2/3 | 3/3 |
| ANG ● | 0/3 (0%) | 0/3 (0%) | 3/3 (100%) |
| MVC * ▲ | 5/5 (100%) | 0/5 (0%) | 0/5 (0%) |
| ANG + ESWT ● | 0/6 (0%) | 2/6 (33,3%) | 4/6 (66,7%) |
| MVC + ESWT | 5/7 (71,4%) | 1/7 (14,3%) | 1/7 (14,3%) |

| WPO8 | | | |
|---|---|---|---|
| Ability | 1/3 | 2/3 | 3/3 |
| ANG | 0/3 (0%) | 0/3 (0%) | 3/3 (100%) |
| MVC ▲ | 4/5 (80%) | 1/5 (20%) | 0/5 (0%) |
| ANG + ESWT ● | 0/6 (0%) | 1/6 (16,7%) | 5/6 (83,3%) |
| MVC + ESWT | 4/7 (57,1%) | 1/7 (14,3%) | 2/7 (28,6%) |

| WPO9 | | | |
|---|---|---|---|
| Ability | 1/3 | 2/3 | 3/3 |
| ANG | 0/3 (0%) | 0/3 (0%) | 3/3 (100%) |
| MVC ▲ | 4/5 (80%) | 1/5 (20%) | 0/5 (0%) |
| ANG + ESWT ● | 0/6 (0%) | 0/6 (0%) | 6/6 (100%) |
| MVC + ESWT | 4/7 (57,1%) | 0/7 (0%) | 33/7 (42.9%) |

| WPO10 | | | |
|---|---|---|---|
| Ability | 1/3 | 2/3 | 3/3 |
| ANG | 0/3 (0%) | 0/3 (0%) | 3/3 (100%) |
| MVC | 3/5 (60%) | 1/5 (20%) | 1/5 (20%) |
| ANG + ESWT | 0/6 (0%) | 0/6 (0%) | 6/6 (100%) |
| MVC + ESWT | 4/7 (57,1%) | 0/7 (0%) | 33/7 (42.9%) |

| WPO11 | | | |
|---|---|---|---|
| Ability | 1/3 | 2/3 | 3/3 |
| ANG | 0/3 (0%) | 0/3 (0%) | 3/3 (100%) |
| MVC | 3/5 (60%) | 0/5 (20%) | 2/5 (40%) |
| ANG + ESWT | 0/6 (0%) | 0/6 (0%) | 6/6 (100%) |
| MVC + ESWT | 3/7 (42,9%) | 1/7 (14,3%) | 3/7 (42,9%) |

| WPO12 | | | |
|---|---|---|---|
| Ability | 1/3 | 2/3 | 3/3 |
| ANG | 0/3 (0%) | 0/3 (0%) | 3/3 (100%) |
| MVC | 3/5 (60%) | 0/5 (20%) | 2/5 (40%) |
| ANG + ESWT | 0/6 (0%) | 0/6 (0%) | 6/6 (100%) |
| MVC + ESWT | 1/7 (14,3%) | 3/7 (42,9%) | 3/7 (42,9%) |

One animal had to be excluded from statistical analysis because the preoperatively recorded data was lost due to a technical error. Animal motivation to participate in the procedure showed some substantial fluctuations over time in our study. Of all rats (n=55),

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34 animals showed no motivation to participate in the grasping test at least once over the entire observation period. In the ANG group and MCV groups 11 animals in each case were reluctant to grasp the bar at least once. The same applied to eight animals in the ANT+ and 4 in the MVC+ groups, respectively. Therefore, these animals had to be excluded from statistical analysis.

Comparison of the remaining 21 animals (**Error! Reference source not found.**) of the ANT (n=3), MVC (n=5), ANT+ (n=6) and MVC (n=7) revealed significant difference regarding the overall grasping ability during the course of the observational period (**Error! Reference source not found.**). While no animal displayed toe flexion until WPO2 in any group, functional recovery was observed to occur fastest in the two groups of animals which underwent median nerve reconstruction with an ANG. Starting from WPO6, all animals in the ANG group had regained the ability to grasp the bar with measurable force. In the ANG+ESET group five out of six animals had regained full motor function, i.e. a grasping rating of 3/3, in WPO8 with the remaining animal displaying this ability starting from WPO9.

Animals which had undergone nerve reconstruction with a MVC but did not receive additional ESWT showed no sign of functional recovery until WPO8. At the end of the observation period two out of five animals had regained grasping ability. While both these animals were able to flex their toes with measurable force the remaining three animals did not show any sign of functional recovery regrading voluntary grasping ability.

In the MVC+ESWT group, first signs of motor recovery became apparent in WPO5 with one animal regaining the ability to grasp the bar without measurable strength. In WPO12 six of the seven animals displayed signs of functional recovery, i.e. a grasping rating of 2/3 or 3/3.

Regarding the evaluation of mean grasping strength as compared to baseline recordings (**Error! Reference source not found.**), no grasping strength was recordable until WPO4 in any group. In WPO4, grasping strengths measured in the ANG group and the ANG+ESWT group were not statistically significant different from each other, while there was still no grasping strength measurable in both groups which underwent nerve repair by means of an MVC. In WPO5, mean grasping strength increased in both the ANG and ANG+ESWT group while animals in both MVC group had still not recovered any grasping strength. One week later, six weeks postoperatively, animals of the ANG group had recovered significantly ($p<0.05$) more grasping strength than those of the MVC+ESWT group. This difference was not statistically significant ($p=0.075$) when comparing the ANG group to the MVC group. The difference of mean grasping strength ratios between the ANG+ESWT and MVC+ESWT group was also not statistically significant ($p=0.058$). In WPO7, rats in the ANG group had recovered significantly ($p<0.05$) greater grasping strength than the rats in both the MVC and MVC+ESWT group, respectively. Eight weeks postoperatively, mean grasping strength in the ANG+ESWT group was significantly ($p<0.05$) higher compared to the MVC group. No statistically significant differences could be observed regarding the ANG or MVC+ESWT group, respectively. In WPO9, animals in the MVC group had still not recovered any grasping strength which was statistically significant ($p<0.05$) compared to the two groups which underwent median nerve repair with an ANG or an ANG+ESWT, respectively. Ten weeks after median nerve reconstruction, rats in all group had recovered measurable grasping strength, but there were no statistically significant differences observable between the groups. The same applied the following postoperative week. At the end of the observation period at WPO12, mean grasping strength ratios were also not statistically significantly different between the four groups.

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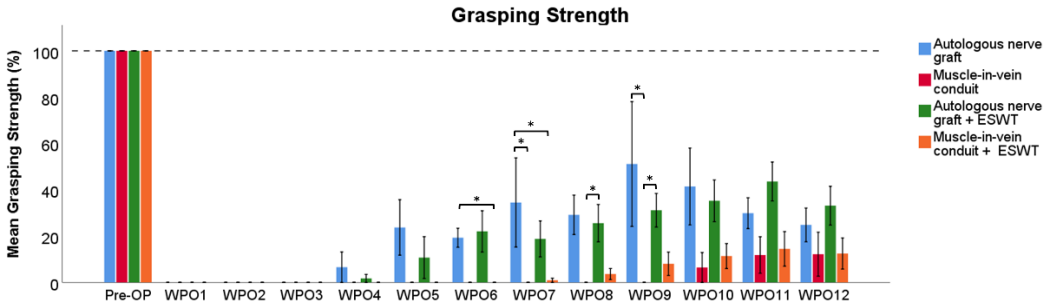


**Figure 2.** Recovery of grasping strength following reconstruction of the right median nerve. Statistical analysis was performed with the non-parametric Kruskal-Wallis test and the Dunn-Bonferroni post-hoc-test. Autologous nerve graft (n=3); Muscle-in-vein conduit (n=5); Autologous nerve graft with postoperative extracorporeal shockwave therapy (n=6); Muscle-in-vein conduit with postoperative extracorporeal shockwave therapy (n=7). WPO: postoperative week; *: $p < 0.05$. Data shown as Mean ± 1 Standard Error of the Mean (SEM).

*CatWalk XT gait analysis*

Computerized gait analysis was used in this study in order to evaluate recovery of sensory and motor function following median nerve resection and immediate reconstruction.

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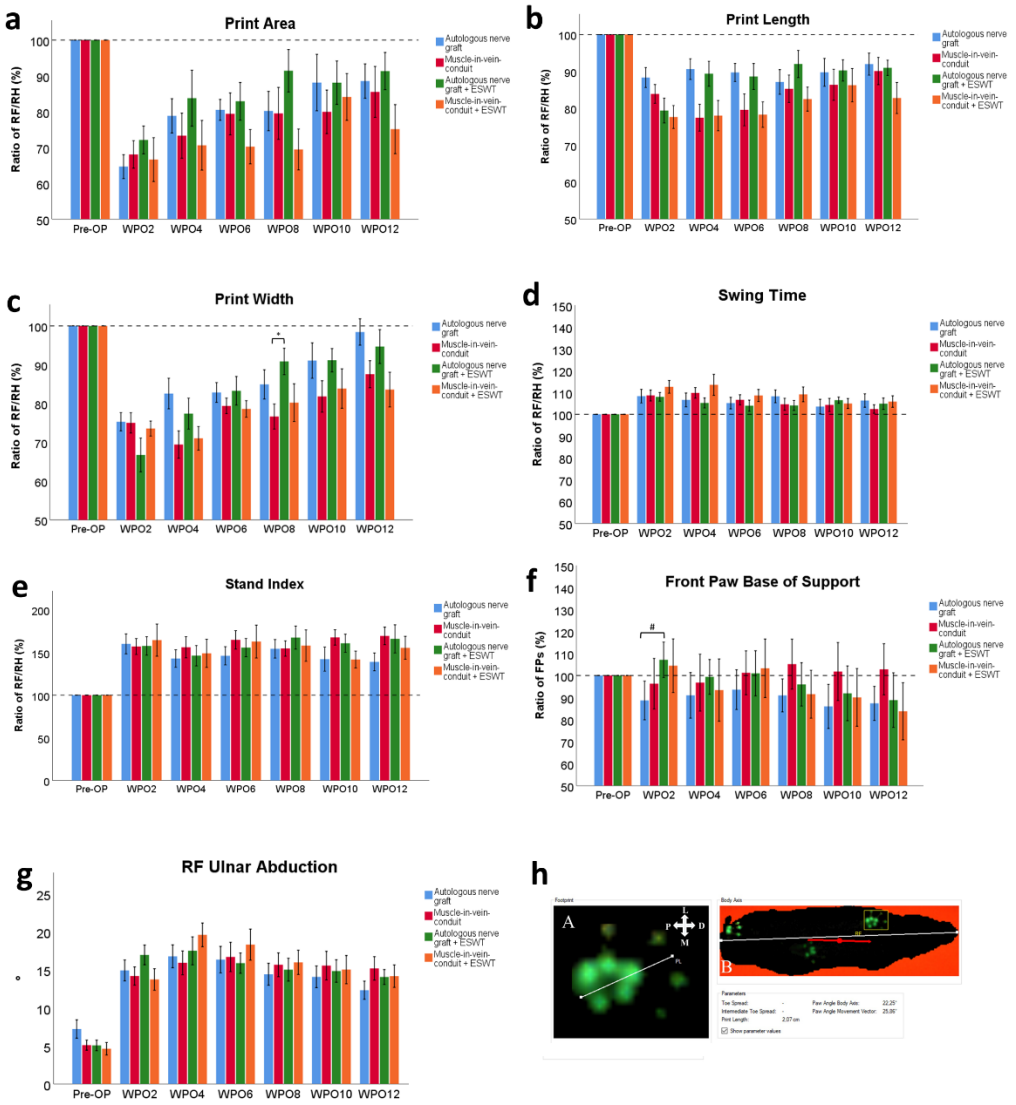


**Figure 1.** Results of computerized gait analysis via CatWalk XT version 10.6 following resection of a 7mm segment of the right median nerve and immediate reconstruction with an autologous nerve graft (n=13); muscle-in-vein conduit (n=16), **autologous nerve graft + ESWT:** autologous nerve graft with postoperative extracorporeal shockwave therapy (n=15), or **muscle-in-vein conduit + ESWT:** muscle-in-vein conduit with postoperative extracorporeal shockwave therapy (n=11). The assessed parameter included: Paw Print Area of the right front paw (**a**), Print Length Ratio of the right front paw (**b**), Print Width Ratio of the right front paw (**c**), Swing Time Ratio of the right front paw (**d**), Stand Index Ratio of the right front paw (**e**), Front Paw Base of Support Ratio (**f**) and Ulnar abduction of the right front paw (**g**) with the schematic of how to assess this latter parameter depicted in figure panel **h**. Statistical analysis was performed with the non-parametric Kruskal-Wallis test and the Dunn-Bonferroni post-hoc-test. For subgroup analysis of identical reconstructive approaches the Mann-Whitney-U-Test was used. Data shown as Mean ± 1 Standard Error of the Mean (SEM). **WPO:** postoperative week, **FPs:** Front Paws, *: $p < 0.05$, #: $p < 0.05$ in subgroup analysis only

Print Area (**Figure 1a**)

There were no significant differences between the four groups regarding the Print Area Ratio RF/RH during the entire course of the 12-week observation period. There was however a trend towards better functional recovery in two groups in which the median nerve was reconstructed with an ANG. The animals which received a MVC and additional ESWT showed a trend towards a lower Print Area Ratio RF/RH. Subgroup analysis of identical reconstructive techniques did not reveal any significant differences.

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Print Length (**Figure 1**b)

Print Length Ratio RF/RH was not significantly different between groups over the entire course of the observational period. In accordance with the course of the Print Area Ratio, a trend towards better functional recovery was apparent in the ANG and ANG+ESWT group especially at WPO4 and WPO6, respectively. Subgroup analysis did also not reveal any significant differences between groups with identical reconstructive technique over the course of the entire observation period.

Print Width (**Figure 1**c)

Print Width Ratio RF/RH was markedly decreased in all groups following segmental median nerve injury. Starting from WPO4 there was a trend towards higher Print Width Ratio RF/RH in the two groups which underwent median nerve reconstruction with an ANG compared to the groups in which a MVC was used. At WPO8, rats which underwent median nerve reconstruction with an ANG and received additional ESWT had a significantly ($p<0.05$) higher Print Width Ratio than those which underwent median nerve reconstruction with an MVC but without additional ESWT.

Swing Speed (data not shown)

Analysis of the Swing Speed Ratio RF/RH did not reveal any marked alterations of this parameter compared to preoperative measurement in any group. No statistical differences were observable between groups either.

Swing Time (**Figure 1**d)

In regard to the Swing Time Ratio RF/RH no statistically significant differences were observable between the four groups at any time point. The parameter was increased in all groups following right median nerve injury and reconstruction. Subgroup analysis did not reveal significant differences between groups which underwent right median nerve repair with the identical reconstructive technique.

Duty Cycle (data not shown)

There were no significant differences detectable between groups in regard to the Duty Cycle Ratio RF/RH (data not shown) over the entire course of the observation period. Subgroup analysis did also not reveal any significant differences between groups which underwent median nerve reconstruction by means of the same reconstructive technique.

Stand Index (**Figure 1**e)

The Stand Index Ratio RF/RH was markedly increased following median nerve resection and immediate reconstruction in all groups. Statistical analysis revealed no significant differences between groups and subgroup analysis of groups with identical reconstructive techniques did also not yield any statistically significant differences between these groups.

FP BoS (**Figure 1**f)

Differences in FP BoS at WPO2 nearly reached statistical significance ($p=0.061$) between ANG-treated animals and animals of the ANG+ESWT group when comparing all groups. In the subgroup analysis of identical reconstructive techniques this difference was highly statistically significant ($p<0.05$).

Ulnar Abduction of the right front paw (**Figure 1**g)

Ulnar abduction of the right front paw, measured as published previously by our group (**Figure 1h**) [39], was not statistically significant different between groups at any

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pre- or postoperative timepoint. This also applied to the subgroup analysis of animals which underwent median nerve reconstruction with an ANG or MVC, respectively.

Appearance of the reconstructed median nerve at WPO12

During the initial surgery we took images of the ANG and MVC we used to reconstruct the right median nerve (**Error! Reference source not found.a + c**). When rats were sacrificed in deep anesthesia twelve weeks postoperatively the reconstructed right median nerve was inspected microscopically to assess the appearance of the regenerated tissue (**Error! Reference source not found.b + d**). These differences were also compared to images taken during the initial surgery. While there were no gross differences observable between identical reconstructive techniques, e.g. ANG vs ANG+ESWT and MVC vs MVC+ESWT, respectively we identified some major distinctive features between animals which underwent median nerve reconstruction with an ANG (**Error! Reference source not found.a + b**) or MVC (**Error! Reference source not found.c + d**). While ANGs at WPO12 were comparable in diameter and length to the grafts sutured between the nerve stumps initially, the MVCs appeared significantly stretched, in some cases reaching lengths of more than 10mm. Additionally, we noticed prominent neuroma formation at the proximal coaptation site in almost all cases. The MVCs were also markedly narrower in their distal segments when compared to the proximal nerve segment. This trend was also observable the more distal parts of the MVC were inspected, reaching smallest diameters at the site of distal nerve coaptation.

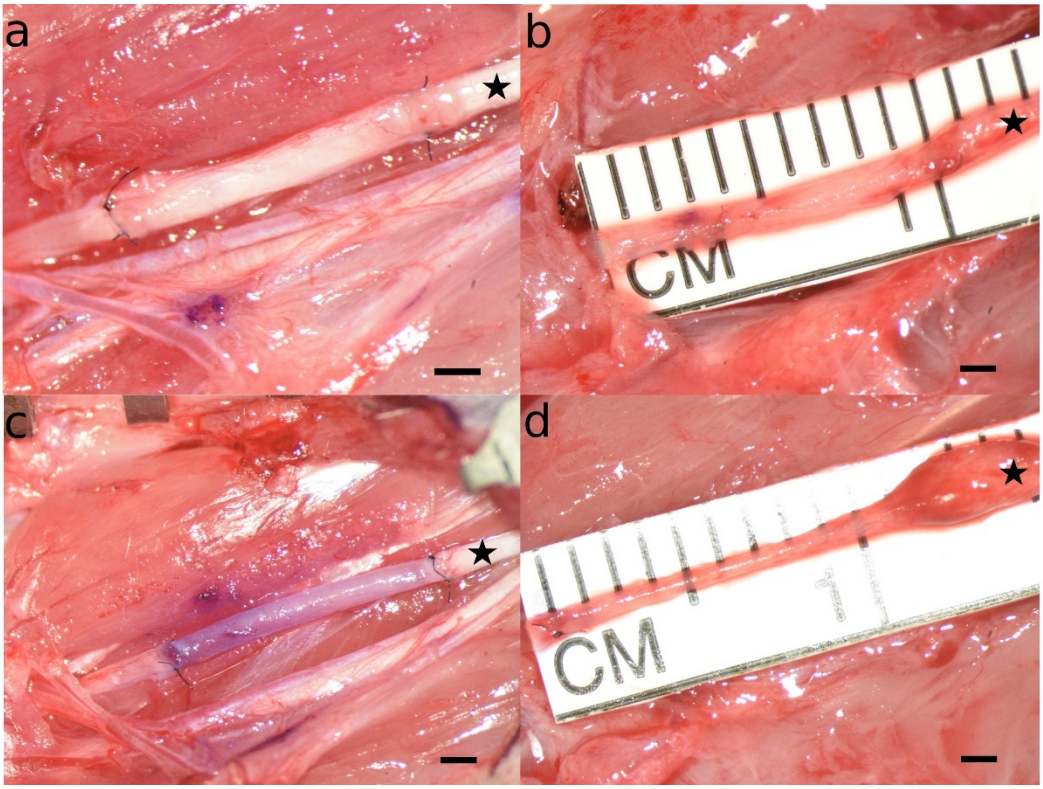


**Figure 4.** Microscopic appearance of autologous nerve grafts (**a + b**) and muscle-in-vein conduits(**c + d**) immediately after nerve reconstruction (**a + c**) and at the timepoint of sacrification twelve weeks after the initial surgery (**b + d**). Note that while Autologous neve grafts at WPO12 were comparable both in length and diameter to the originals grafts sutured to the stumps of the median nerve during the initial surgery, muscle-in-vein-conduits appeared markedly thinner and stretched at the time point of sacrification as compared to the initial surgery. Additionally, a prominent coaptation neuroma was observable at the proximal coaptation site in almost all cases a muscle-in-vein conduit was used for nerve reconstruction. The proximal part of the reconstructed nerve is marked with an asterisk. Scale bar = 1 mm.

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Electrophysiological evaluations

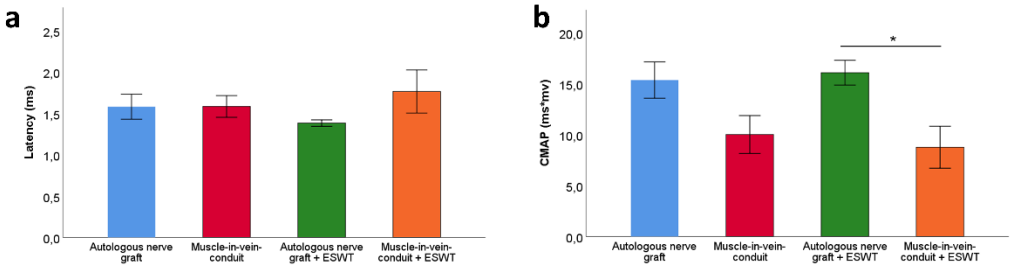


**Figure 5.** Distal motor latency (**b**) and compound muscle action potential area (**b**)of the reconstructed right median nerve 12 weeks following resection of a 7mm segment of the right median nerve and immediate reconstruction with an autologous nerve graft (n=14); muscle-in-vein conduit (n=11), **autologous nerve graft + ESWT:** autologous nerve graft with postoperative extracorporeal shockwave therapy (n=15), or **muscle-in-vein conduit + ESWT:** muscle-in-vein conduit with postoperative extracorporeal shockwave therapy (n=8). Results of the electrophysiological evaluations were compared between groups with the non-parametric Kruskal-Wallis test and the Dunn-Bonferroni post-hoc-test. Data shown as Mean ± 1 Standard Error of the Mean (SEM). **CMAP:** Compound muscle action potential. ***:** $p < 0.05$.

Statistical comparison of distal motor latencies (**Error! Reference source not found.a**) and CMAP areas (**Error! Reference source not found.b**) at WPO12 revealed no statistically significant differences between groups in regard to distal motor latency. Regarding CMAP area, there was a trend towards higher values observable in the two groups which underwent median nerve reconstruction with an ANG. Values were significantly ($p<0.05$) higher in the ANG+ESWT group when compared to the MVC+ESWT group. CMAP values in the ANG and MVC group ranged between those two, but there were no statistically significant differences observable.

Wet muscle weight

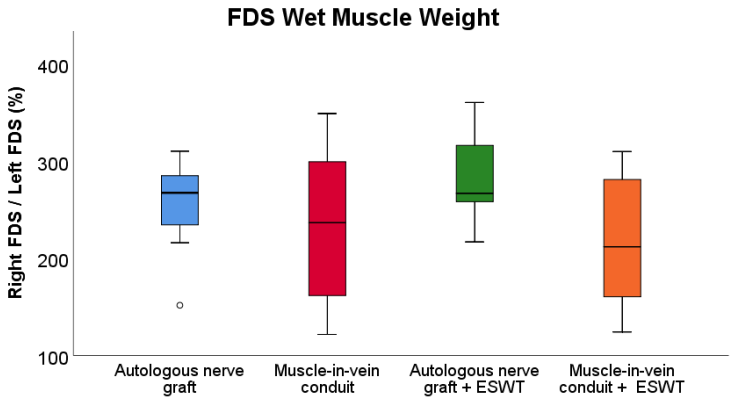


**Figure 6.** Comparison of wet muscle weight ratio of the right and left flexor digitorum superficialis muscle at postoperative week 12 following bilateral resection and unilateral reconstruction of the right median nerve. Autologous nerve graft: n=14; muscle-in-vein-conduit: n=15, **autologous nerve graft + ESWT**: autologous nerve graft with postoperative extracorporeal shock wave therapy (n=15); **muscle-in-vein-conduit + ESWT:** muscle-in-vein conduit with postoperative extracorporeal shock wave therapy (n=11). Statistical analysis was performed with the non-parametric Kruskal-Wallis test and the Dunn-Bonferroni post-hoc-test. For subgroup analysis of identical reconstructive approaches the Mann-Whitney-U-Test was used. **FDS:** Flexor digitorum superficialis muscle. Data shown as Mean ± 1 Standard Error of the Mean (SEM).

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Despite a trend of higher FDS muscle weight ratios (**Error! Reference source not found.**) in both the ANG and ANG+ESWT group, respectively, there were no statistically significant differences ($p = 0.05$) observable between the four groups. FDS muscle weight ratios in the MVC and MVC+ESWT group were lower than in the ANT and ANT+ESWT group with a trend for lowest values in the groups of rats which received an MVC+ESWT.

Histological evaluations

The schematic indicating the histological sections of the reconstructed median nerve taken at WPO12 is depicted in ***Error! Reference source not found.***.

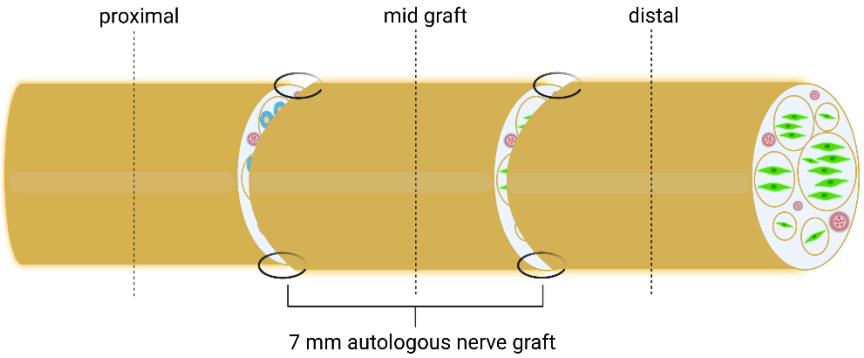


**Figure 7.** Schematic of the repaired median nerve with histological sections and their respective localization indicated as proximal, start graft, mid graft and distal. Dotted lines indicate histological cutting planes [49].

Number of axons

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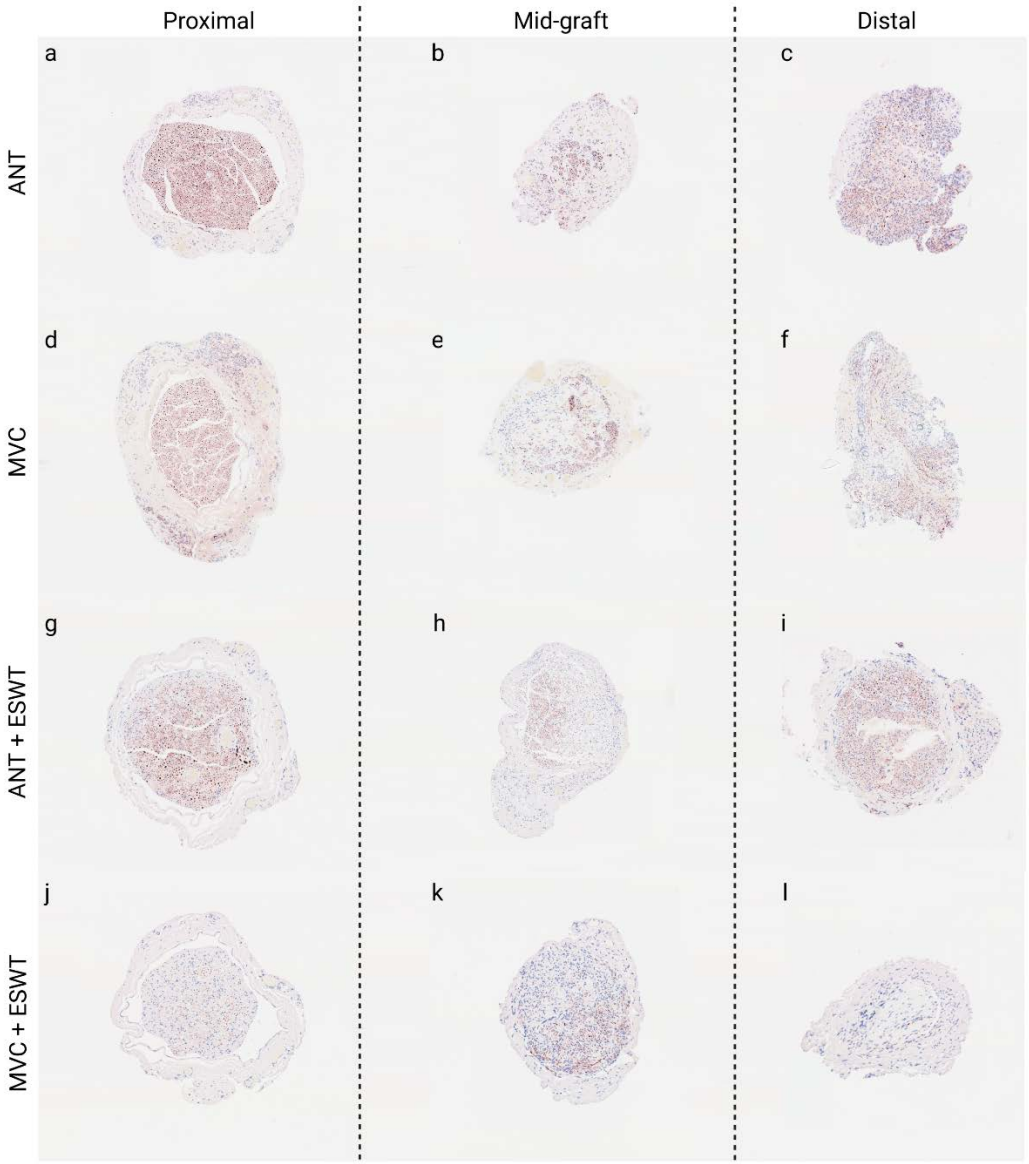


**Figure 8.** Representative photomicrographs of anti-**neurofilament** stained consecutive cross-sections through the regenerated tissue in the proximal nerve segment (**a,d,g,j**), middle of the nerve graft (**b,e,h,k**) and distal nerve graft (**c,f,i,l**) at 12 weeks post-surgery. **a-c:** Autologous nerve graft; **d-f:** muscle-in-vein-conduit; **g-i:** autologous nerve graft + extracorporeal shockwave therapy; **j-l:** muscle-in-vein-conduit + extracorporeal shockwave therapy. **Scale bar** = 200µm.

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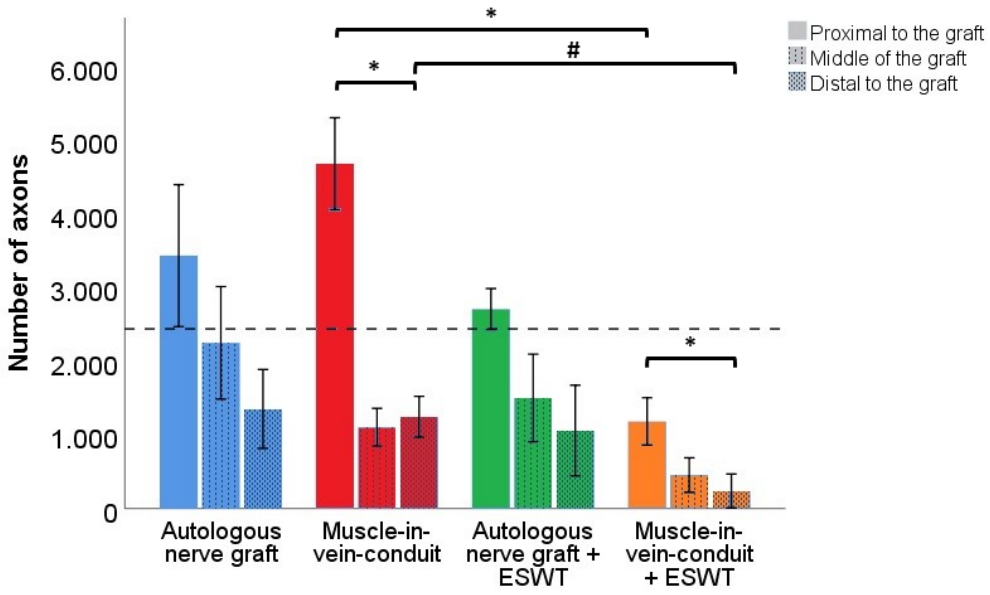


**Figure 9.** Number of axons in different segments of the reconstructed right median nerve 12 weeks following resection of a 7mm segment of the right median nerve and immediate reconstruction with an autologous nerve graft (n=5); muscle-in-vein conduit (n=6), autologous nerve graft + ESWT: autologous nerve graft with postoperative extracorporeal shockwave therapy (n=4), or muscle-in-vein conduit + ESWT: muscle-in-vein conduit with postoperative extracorporeal shockwave therapy (n=4). The dashed line indicates the number of axons in an uninjured median nerve at the level of the mid humerus (2442 ± 19) Statistical evaluation was performed with the non-parametric Kruskal-Wallis test and the Dunn-Bonferroni post-hoc-test. For subgroup analysis of identical reconstructive approaches the Mann-Whitney-U-Test was used. Repeated measurements of the same group were compared by means of the non-parametric Friedman test. Data shown as Mean ± 1 Standard Error of the Mean (SEM). ***:** $p < 0.05$, **#:** $p < 0.05$ in subgroup analysis only.

Representative photomicrographs of anti-neurofilament stained cross-section are shown in **Error! Reference source not found.**. Statistical comparison (**Error! Reference source not found.**) of proximal nerve segments revealed statistically significant ($p<0.05$) lower axon numbers in the proximal nerve segments of MVCs which were treated with ESWT postoperatively (1184 ± 321) when compared to MVCs which received no additional treatment (4683 ± 624). In accordance with this observation, more axons were found in the proximal nerve segments of untreated ANGs (3436 ± 963) than in the ANG+ESWT group (2710 ± 278), but this trend was not statistically significant.

Axon numbers in the mid-graft segments of the ANG group (2251 ± 764), MVC group (1106 ± 255), ANG+ESWT group (1502 ± 595) and MVC+ESWT group (456 ± 233) were not statistically significantly different from each other. The same applied to the axons numbers in the distal nerve segments. Axon numbers were highest in the ANG group (1354 ± 535) followed by the MCV group (1248 ± 277) and lowest in the MVC+ESWT (239± 230) group with the ANG+ESWT group (1060 ± 615) in between.

Subgroup analysis of identical reconstructive approached revealed significant lower number of axons in both the proximal as well as distal nerve segments of animals which underwent median nerve reconstruction with a MVC and additional ESWT. When we compared the number of axons within the same reconstructed nerve for each group, significant lower numbers were found in the distal nerve segments of MVC and MVC+ESWT treated animals when compared to the counts in the proximal nerve segment. No significant differences were found in case of the other two groups.

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**Number of blood vessels**

Representative photomicrographs of anti-CD31 stained cross-section of the reconstructed median nerve are shown in **Supplementary figure 3**. **Figure 2** displays the evaluation of the number of blood vessels in these segments.

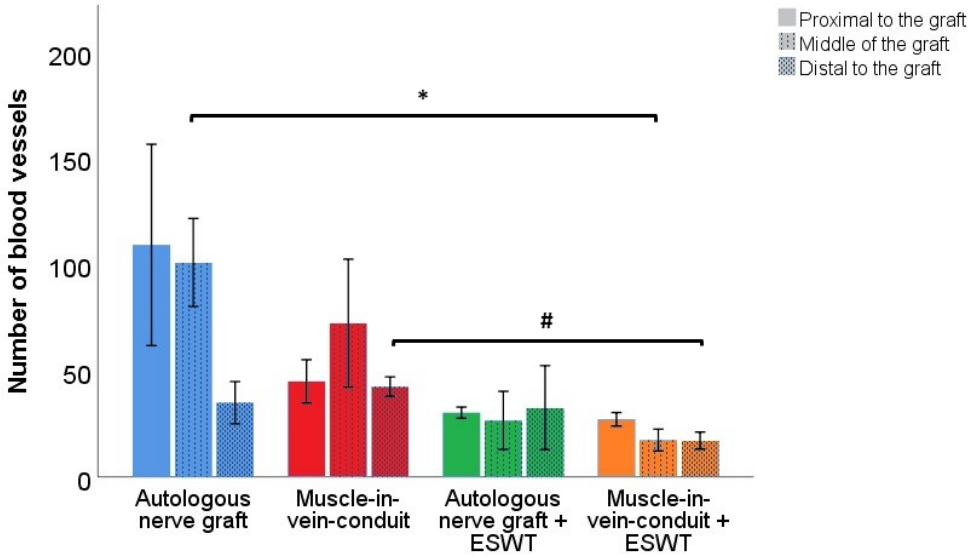


**Figure 2 –** Number of blood vessels in different segments of the reconstructed right median nerve 12 weeks following resection of a 7mm segment of the right median nerve and immediate reconstruction with an autologous nerve graft (n=4); muscle-in-vein conduit (n=5), autologous nerve graft + ESWT: autologous nerve graft with postoperative extracorporeal shockwave therapy (n=3), or muscle-in-vein conduit + ESWT: muscle-in-vein conduit with postoperative extracorporeal shockwave therapy (n=5). Statistical evaluation was performed with the non-parametric Kruskal-Wallis test and the Dunn-Bonferroni post-hoc-test. For subgroup analysis of identical reconstructive approaches the Mann-Whitney-U-Test was used. Repeated measurements of the same group were compared by means of the non-parametric Friedman test. Data shown as Mean ± 1 Standard Error of the Mean (SEM). ***:** $p < 0.05$, **#:** $p < 0.05$ in subgroup analysis only

Blood vessel numbers in the proximal segments of reconstructed median nerves were not statistically significant different between animals of the ANG (109 ±47), MVC (66 ± 23), ANG+ESWT (30 ±3) or MVC+ESWT group (27 ±3), respectively. The same applied to the number of blood vessels in the distal segment of the reconstructed nerve. While numbers were lowest in the MVC+ESWT group (17 ±4), counts in the ANG (35 ±10) and ANG+ESWT (33 ±20) group were almost the same. Most blood vessels could be found in the MVC group (43 ±5) at this level.

Regarding the number of blood vessels at the mid-graft level, significant differences were found between animals of the ANG group (101 ±21) and MVC+ESWT group (17 ±5). There were no statistically significant differences observable regarding the ANG+ESWT (27 ±14) or MVC group (72 ±30).

Subgroup analysis of identical reconstructive approaches by means of the Mann-Whitney-U-Test revealed a statistically significant ($p<0.05$) lower number of blood vessels in the distal nerve segments of MVC+ESWT-treated animals as compared to the group of animals which received a MVC without additional ESWT.

Comparison of blood vessel numbers within different nerve segments of each group revealed no statistically significant differences.

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**Number of lymphatic vessels**

Figure 3 displays representative photomicrographs of anti-podoplanin stained consecutive cross-sections of the reconstructed median nerve.

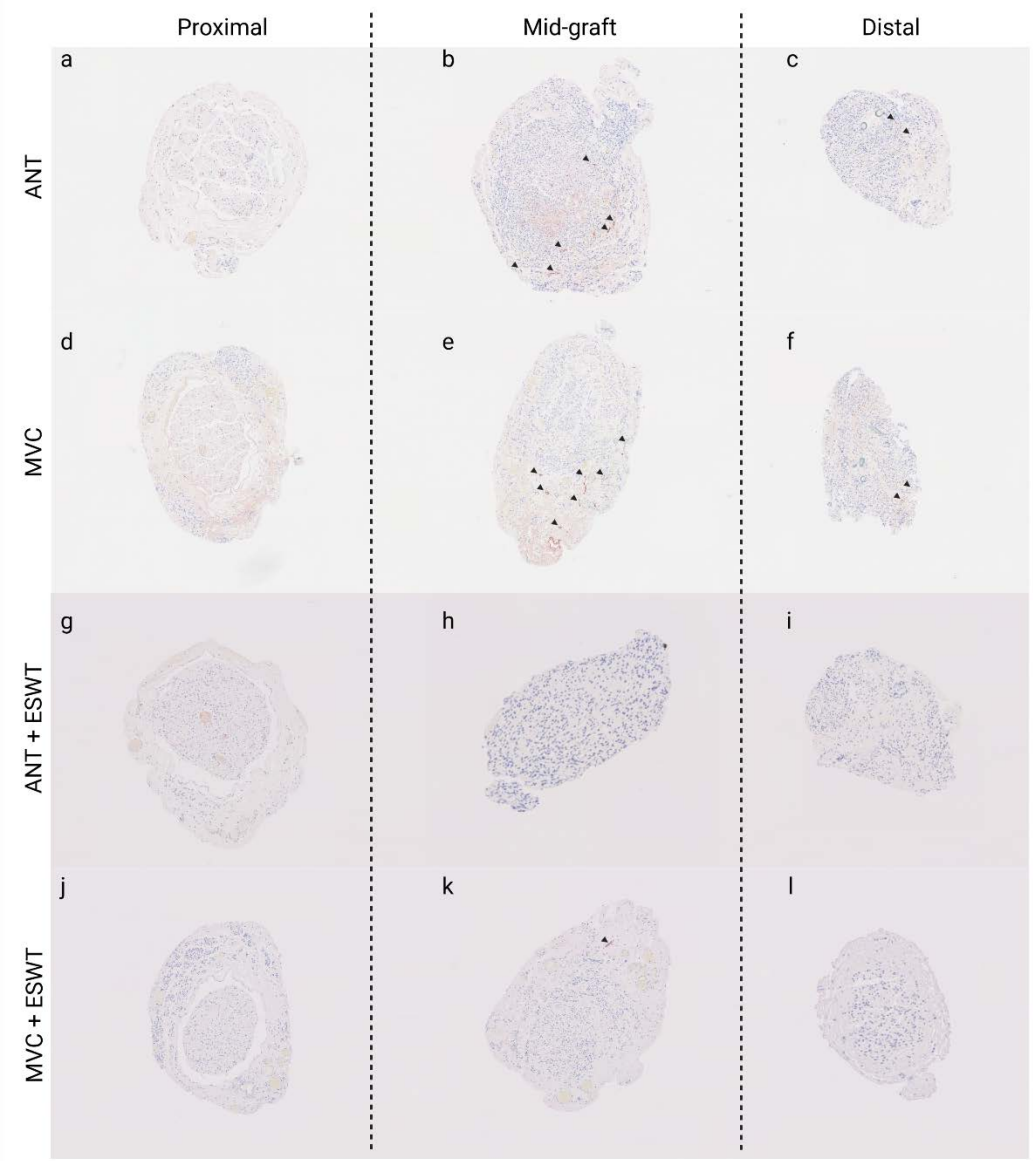


**Figure 3 -** Representative photomicrographs of anti-**podoplanin** stained consecutive cross-sections through the regenerated tissue in the proximal nerve segment (**a,d,g,j**), middle of the nerve graft (**b,e,h,k**) and distal nerve graft (**c,f,i,l**) at 12 weeks post-surgery. **a-c:** Autologous nerve graft; **d-f:** muscle-in-vein-conduit; **g-i:** autologous nerve graft + extracorporeal shockwave therapy; **j-l:** muscle-in-vein-conduit + extracorporeal shockwave therapy. **Scale bar** = 200µm. Podoplanin-positive stained lymphatic vessels are indicated by arrowheads.

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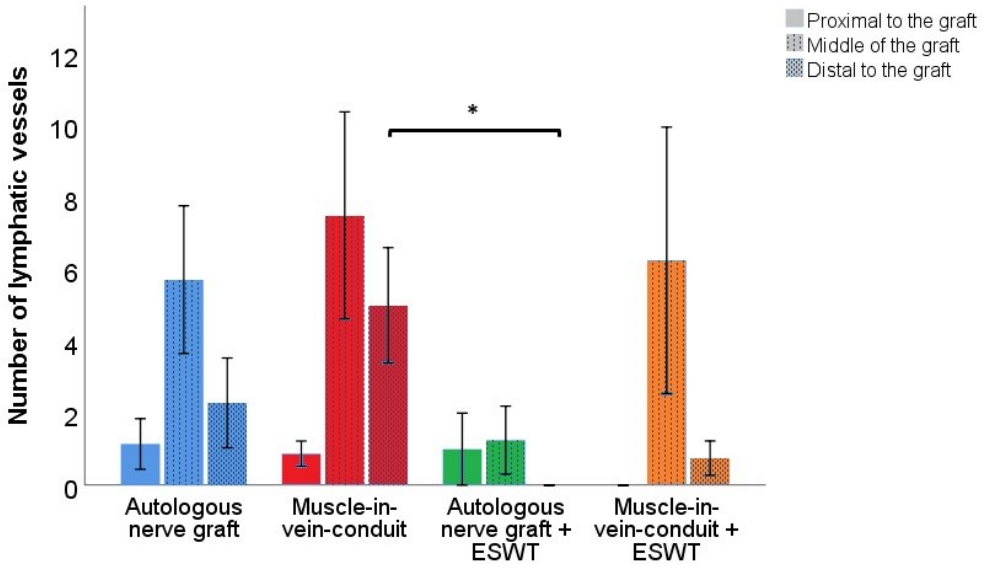


**Figure 4 –** Number of lymphatic vessels in different segments of the reconstructed right median nerve 12 weeks following resection of a 7mm segment of the right median nerve and immediate reconstruction with an autologous nerve graft (n=8); muscle-in-vein conduit (n=8), autologous nerve graft + ESWT: autologous nerve graft with postoperative extracorporeal shockwave therapy (n=5), or muscle-in-vein conduit + ESWT: muscle-in-vein conduit with postoperative extracorporeal shockwave therapy (n=4). Statistical evaluation was performed with the non-parametric Kruskal-Wallis test and the Dunn-Bonferroni post-hoc-test. For subgroup analysis of identical reconstructive approaches the Mann-Whitney-U-Test was used. Repeated measurements of the same group were compared by means of the non-parametric Friedman test. Data shown as Mean ± 1 Standard Error of the Mean (SEM). *: $p < 0.05$

Statistical comparison of the number of lymphatic vessels in different segments of the reconstructed median nerve between groups (**Figure 4**) revealed no statistically significant differences between groups in regard to the proximal and mid-graft segment.

In the proximal nerve segment lymphatic vessel numbers in the ANG group (1.14 ± 0.70), MVC group (0.88 ± 0.35) and ANG+ESWT (1.00 ± 1.00) group were almost identical whereas no lymphatic vessels could be identified within the proximal nerve segment of MVC+ESWT-treated animals.

Number in the mid-graft segment were highest in the MVC group (7.50 ±2.87), followed by the MVC+ESWT group (6.25 ±3.70), ANG group (5.00 ±1.91) and ANG+ESWT group (1.00 ±0.77).

Statistically significant ($p<0.05$) differences in lymphatic vessel counts could be identified in the distal nerve segments of ANG+ESWT (0) and MVC-treated (5.00 ±1.60) animals. There were no statistically significant differences regarding number in the ANG (2.00 ±1.11) and MVC+ESWT group (0.75 ±0.47), respectively.

Subgroup analysis of identical reconstructive approaches by means of the Mann-Whitney-U-Test revealed no statistically significant differences.

When the number of lymphatic vessels within different nerve segments of each group were compared with the Friedman Test also no statistically significant differences were found.

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## 4. Discussion

This study's main hypothesis was that regeneration of the murine median nerve following reconstruction with ANGs, i.e. the gold standard method, or MVCs, i.e. non-neural tissue, can be enhanced by a single postoperative application of low-energy defocused ESWT. The proregenerative effects of ESWT have been shown in the context of various musculoskeletal and neurological diseases, including carpal tunnel syndrome [50-53], spinal cord injury [54-58] and PNI in vivo [17,19,20,27,58-60]. Of note, to the best of our knowledge all PNI studies were performed in the sciatic nerve model of the rat and four of the seven studies we retrieved featured a sciatic crush injury [19,58-60], e.g. axonotmesis. In regard to the sciatic nerve injury model it must be noted that this model has some significant disadvantages, most noteworthy the development of neuropathic pain and automutilations in case of neurotmesis injuries and difficulties to assess functional recovery by means of walking track or gait analysis [28]. Considering the severity of the nerve injury, it must be noted that while crush type nerve injuries in humans are likely to recover ad integrum even in the absence of any surgical or pharmacological treatment, this occurs even faster in rodents given the profound differences in neurobiology and speed of axonal regeneration between species [61]. Neurotmesis injuries however are likely to show incomplete or even insufficient functional recovery even in case of optimal surgical treatment [47]. We therefore reasoned it to be essential to investigate the effects of ESWT to enhance functional recovery following neurotmesis of the rat median nerve. Given that the use of nerve autografts to reconstruct peripheral nerves is limited by their restricted availability and resulting donor site morbidity we also deemed it indispensable to evaluate whether positive effects of ESWT can also be observed following median nerve reconstruction with non-neuronal tissue, e.g. MVCs.

First of all, the promising reports of studies evaluating functional recovery following nerve reconstruction with MVCs in humans [43,44,62] could not be reproduced in our study. This is in line with the findings of others who have shown that functional recovery following nerve reconstruction by means of MVCs were markedly inferior in comparison to autologous nerve grafting in rodent models [38,63]. In one of our previous works we have already suggested that this might be related to a higher number of proteases in the murine genome, resulting in increased protein turnover and therefore accelerated degeneration of muscle fibers within the MVC which in turn might hamper axonal regeneration due to a decrease in MVC intraluminal diameter [45]. This was also supported by the intraoperative findings we observed at WPO12 in regard to the diameter of the distal segments of MVCs.

More importantly, despite some trends for favourable functional outcome in the group of animals receiving ESWT in addition to nerve reconstruction with an ANG, our study's results do not support the positive reports of other preclinical works regarding the neuroregenerative effects of ESWT both on peripheral nerves [17,19,27,59,64] and the spinal cord [55,56,58]. The results of the grasping test did not verify a significant positive effect of ESWT on functional recovery following reconstruction of the median nerve with either ANGs or MVCs. Interpretation of this test's results was further hampered by the high number of animals which had to be excluded from statistical analysis due to limited motivation to participate in the assessment, a problematic observation which has also been reported by other authors and might relate to unpleasant sensations experienced by the animals when held at the tail [38,46].

The electrophysiological evaluations at the end of our observation period were not indicative of any significant direct proregenerative effect of a single ESWT application following median nerve reconstruction. Interestingly, these measurements revealed a significantly lower FDS CMAP amplitude area in rats which underwent nerve repair with an MVC+ESWT as compared to ANG-treated animals which also received postoperative ESWT. As no other significant differences were observable between groups, this indicates either a slightly pro-regenerative effect of ESWT following autologous nerve grafting, a

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disadvantageous effect of ESWT in case of nerve repair with MVCs, or both. The wet muscle weights of the FDS revealed a similar trend and did also emphasize the superiority of nerve repair with ANGs as compared to MVCs as reported by us and other authors [38,45,63,65].

Computerized gait analysis with the CatWalk device showed a trend for better functional recovery in rats which underwent nerve repair with ANGs in comparison to MVCs, too. Interestingly, analysis of paw print dimensions, i.e. Print Area, Print Length and Print Width hinted towards improved recovery in rats of the ANG+ESWT group, especially in regard to Print Area Ratio. Additionally, ANG+ESWT treated animals had significantly higher Paw Width Ratio than those which received an MVC, supporting our theory that ESWT treatment exert positive effects on nerve regeneration and functional recovery after autograft repair. The observed increase in Swing Time and Stand Index Ratio following segmental median nerve injury and reconstruction are in line with what we have described previously and emphasize the value of these two parameters, especially the Stand Index Ratio to assess functional deficits following nerve median nerve neurotmesis [39]. Interestingly our subgroup analysis of identical reconstructive approaches showed a significant difference in Front Paw (FP) Base of Support between the ANG and ANG+ESWT group in WPO2. As most parameters of gait, changes in BoS can be induced by several different factors, e.g. an increase in BoS can account for an unstable gait following central nervous lesions [66-68] and a decreased BoS was reported after sciatic nerve neurotmesis in rats [69] whereas it remained not significantly changed following neurotmesis of the rat femoral nerve [33] and median nerve [39]. This might on the one hand be related to relatively high functional deficit following sciatic nerve neurotmesis in contrast to median and femoral nerve neurotmesis [33,39]. In this context early work involving use of the CatWalk device in a rat model of sciatic nerve injury postulated that the observed in BoS in mainly related to a separate motor dysfunction rather that an adaptive response to other functional losses [70]. However, one could also assume that a significant loss of innervated, i.e. sensate plantar paw surface following peripheral nerve injury (PNI) is likely counterbalanced by placing the affected paw and its contralateral counterpart closer together to account for this functional loss. Additionally, the experience of mechanical allodynia during ambulation might also influence the BoS whereas in our study one would expect that this symptoms at WPO2 are most likely caused by collateral sprouting of intact adjacent peripheral nerves in the paw, e.g. the ulnar nerve into the original territory of the median nerve[71]. In our opinion one should also consider the experience of local pain at the operation site as a likely mechanism and a study published in 2018 reported an increase in FP BoS following nerve reconstruction with a conventional nerve flap as opposed to a decrease in FP BoS following median nerve excision, autograft repair and noteworthy sham surgery [72]. As the exact reasons for the significant difference in FP BoS in WPO2 remain to be elucidated in detail, we postulate that this could be related to direct positive effects of ESWT on wound healing on the site of operation [73]. Additionally, it was reported that ESWT induces selective loss of unmyelinated , i.e. nociceptive nerve fibers with potential analgesic effects due to selective denervation of target organs. [74-76]

Regarding quantification of axons within the reconstructed median nerve at WPO12 two main findings require discussion. First of all, we observed a significant decrease in axon numbers in MVCs when comparing the proximal nerve sections with the distal ones. This finding is in total accordance with our and others' observation that axonal regeneration through MVCs is inferior in comparison to ANGs, most likely because axonal regrowth is hindered in case the muscle fibers within the MVC are degraded before the regrowing axons have reached the distal segment of the MVC [38,45,63] as observable by the narrowing of distal MVC segments in our study. In addition, extensive formation of coaptation neuroma at the proximal repair site was reported by the beforementioned authors and was also observable at WPO12 in our study. It has been emphasized that in order to achieve the best possible result when performing nerve reconstruction with an

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MVC is essentially, to pull the nerve stumps into the MVC rather than just coaptating them to the proximal and distal stump [43,44]. However, this is more difficult and technically challenging in a rat model due to the small diameter of the harvested veins used to fabricate the MVC [45]. Second, we observed a significant smaller number of axons in the proximal and distal nerve segments of MVC+ESWT treated animals in comparison to the MVC group. This finding also points towards disadvantageous, i.e. regeneration-hindering effects of ESWT in the context of nerve repairs with MVCs.

The role of vascularization and neo-angiogenesis and their respective assessment has gained increasing attention recently [72,77-88]. In our study significant lower numbers of blood vessels were found in the distal nerve segments of MVCs which were treated with ESWT when compared to rats of the MVC group. In accordance with the previous paragraph these findings provide evidence that the observed effects of ESWT on peripheral nerve regeneration in our study, especially in case of the MVCs, are at least partially related to hindered neovascularization of the regenerating nerve. As ESWT treatment was shown to exert proangiogenic effects in several other studies [21,22,57,64,89-91] the underlying reasons for the observations in our study remain to be elucidated.

Our study is also among the first [49,77,92,93] to shine of a light on the involvement of lymphatic vasculature and lymphangiogenesis, respectively in peripheral nerve repair and regeneration. Interestingly, we found that the number of lymphatics was drastically increased in both ANGs and MCVs following median nerve reconstruction. These vessels were especially prominent in the middle portions of the reconstructed nerve segments. Saffari et al. have recently shown that hemangiogenesis in peripheral nerve regeneration occurs from both stumps of the original nerve but primarily from the proximal one [86]. In our study we observed a relatively higher number of lymphatic vessels in the middle grafts segments of both MVCs and ANGs. We hypothesize that this observation can be explained by a study recently published by our group. In this work we have shown for the first time, that Schwann cells induce apoptosis of lymphatic endothelial cells when co-cultured in vitro via extended filopodia-like protrusions [49]. While this on the one hand explains the absence of lymphatic vessels inside uninjured murine peripheral nerves, these findings can be further extrapolated to the findings in the study at hand. As Schwann cells were shown to induce apoptosis of lymphatic endothelial cells in vitro we hypothesize that the reduced numbers of lymphatic vessels in the proximal and distal segments of ANGs are related to the presence of Schwann cells which migrate into the reconstructed nerve from both the proximal and distal nerve stump [94], inducing apoptosis of lymphatic vessels in this areas. The higher number of lymphatic vessels in MVCs can be explained by the fact that these contain fewer Schwann cells in addition to pro-angiogenetic effects exerted by the vein component of MVCs [95,96]. Although it has been recently hypothesized [92] and later shown [93] that lymphangiogenesis plays an important role in peripheral nerve regeneration following axonotmesis our results also indicate that higher numbers of lymphatic vasculature do not necessarily lead to better functional recovery as illustrated by the MVC+ESWT group in our study. Given the complexity of the peripheral nervous system and the intricate interplay of its components it is reasonable to assume that “the more the better” does not necessarily apply in this context. Although not statistically significant we observed a trend towards lower numbers of lymphatic vessels in the distal nerve segments of ESWT-treated ANGs as compared to the ANG-group which is in accordance with what we observed regarding the number of blood vessels in the reconstructed median nerves. As aforementioned, ESWT was shown to exert major pro-angiogenetic effects in vivo [22,91] making interpretation of these results more difficult. However, Hausner et al. showed that ESWT did not increase the number of blood vessels in a sciatic nerve autografting model [27]. Circling back to potential interplay of Schwann cells and lymphatic vasculature in vivo, we hypothesize that the lower number of lymphatic vessels might be explained by direct effects of ESWT on Schwann cells which in turn indirectly affects lymphatic endothelial cells. As it was shown that *ex vivo* ESWT-treated Schwann cells showed increased proliferative activity and –

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upon respective inductive cues - expression of myelin-associated phenotypic markers [25] this might exert negative effects on lymphatic endothelial vasculature cells in vivo. Given the few published reports [17,24,25,97] addressing the effects of ESWT on Schwann cells we advise for further studies to elucidate the underlying reasons for this phenomenon.

Our study bears several limitations. First of all, the majority of animals had to be excluded from statistical analysis of the grasping test. This was mainly because the animals showed fluctuating and reduced motivation to participate in repeated measurement over the course of the postoperative observation period. This problem has been reported by other authors in the past [38] and is especially concerning when Lewis rats are used in this test, which is still considered the gold standard method for evaluation of functional recovery in rat models of median nerve injury [36]. Given the reduced number of animals eligible for further statistical comparison of grasping strength and grasping ability, results of these comparison should be interpreted with outmost caution. Second, while our study pioneers a standardized application of ESWT to promote peripheral nerve repair via a 3D-printed holder, the ideal application intensity and frequency of ESWT remains to be elucidated in further studies. This might have resulted in significantly higher energies on the target tissue than conventional, free-hand application of ESWT and subsequent negative effects on axonal regeneration. As higher energy ESWT is more effective in regard to the achieved treatment effects so are the side effects, e.g. the aforementioned destruction of unmyelinated nerve fibers [98] and very high intensities are almost certainly more harmful than regeneration-promoting [22].

## 5. Conclusions

Our study investigated a novel application method for ESWT on axonal regeneration as well as the effects of ESWT on the formation of blood and lymphatic vessels in the regenerating nerve after autologous nerve grafting or muscle-in-vein conduit repair. A single postoperative application of defocused low-intensity extracorporeal shock wave therapy did not significantly enhance neuroregeneration in a rat model of segmental median nerve injury but decreased the number of blood and lymphatic vessels within the regenerated nerves. Rats treated with MVCs showed worse functional recovery than those treated with an ANG.

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**Institutional Review Board Statement:** The experimental protocol was approved in advance by the Animal Protocol Review Board of the City Government of Vienna (Approval number: MA58-421715-2019-16). All procedures were carried out in full accord with the Helsinki Declaration on Animal Rights and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

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