# Prolonging Nerve Grafts Using Chemical Extracted Muscle-in-vein with *Vein Window* Method

## Chemical acellular nerve grafts

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*Nerve injuries are a common pathology in hand trauma. The consequences are drastic both for patients and doctors/medical system. In many cases direct coaptation is impossible. A nerve graft should be used in the case of a neuroma, trauma or tumor, for restoration of nervous influx. The aim of this study is demonstrate that by grafting restant nerve stumps with muscle-in-vein nerve grafts we obtain good result in terms of functional and sensibility recovery and also our method ‚window-vein' is a good way of prolonging nerve grafts. The method of study is experimental. We worked in the laboratory in optimal conditions for carrying out of muscles-in-vein nerve grafts (nerve grafts size 1.5 $cm^{-3}$ cm). We used acellular muscle grafts with the chemical extraction method.The study was conducted on experimental animals (Wistar male rats).We used 30 experience animals in 3 equal groups (classical group and muscle-in-vein nerve grafts-2 nerve grafts of 1,5 cm central sutured and the third group with muscle-in-vein nerve grafts, window-vein method, 3 cm). At 4 and respectively 6 weeks postoperative at the quality tests we observed the progress with the footprint test. The operated hind in comparison with the healthy hind was 86% recovered and similar with classic nerve grafts. Quantitatively the number of regenerated axons in the group with muscle-in-vein nerve grafts was significant bigger in comparison with the classical group (15%).The method using muscle-in-vein nerve graft with windows-vein it's a good alternative for nerve grafting in comparison with classical nerve grafting. When the local possibilities are limited, this method is good for prolonging the grafts. The relationship between cost and benefit in this case it's an advantage because we use the local resources of the affected area. The motor results of nerve grafting ingroup 2 in comparison with group 3 were similar and in some cases better in group 1. Grafting with MVNG offers a better alternative for donor site regeneration in comparison with classical nerve grafts. This method is useful to prolong nerve grafts without adding morbidity.*

*Keywords: nerve grafting, regeneration, axons, window-vein, acellular*

Peripheral nerve injuries are a common entity in the treatment of hand disorders. They are most commonly related to direct mechanical trauma, and can be associated with other soft and bone tissue lesions [1)].

Transection of axons implicates in morphologic and metabolic changes throughout the whole length of the neuron. From the moment of injury, the cell body is signalized and the regeneration process starts [2]. While the distal nerve stump undergoes Wallerian degeneration, the proximal stump retracts and the Schwann cells undergo apoptosis [3]. The axon sprouts and a growth cone is formed at the tip of each sprout, interacting with activated and proliferating Schwann cells [4]. The nerve injury itself results in decreasing of motoneurons in the spinal cord. In an experimental study with Wistar rats, the number of motoneurons labeled in the spinal cord decreased an average of 20% due to an isolated tibial nerve injury, with no technical interference by the surgeon [5].

Exploring peripheral nerve injuries was since the early medicine a controversy issue. In our days the most frequent problem is nerve repairing and functional outcome. During time, different types of nerve grafting had success. The most common is allograft with contralateral sural nerve. But in this situation the local possibilities become limited and it adds morbidity. That's how the need for safe grafting appeared [6]. Muscle-in-vein method is used since the early 2000s with good results in specific clinical cases. Battiston et al (2000) published their result on 21 patients with a range of success of 85%. The length of the nerve graft was from 0.5 to 6 cm. Later, in other clinical cases it was observed that grafts longer than 3-4 cm have the best functional outcome in comparison with longer than 5 cm nerve grafts. This gives the hypothesis that we need to find out another convenient method of peripheral nerve grafting. The functional results are comparative with direct coaptation, the cost are small and the second donor site morbidity is avoided [7].

Sometimes the local possibilities of nerve grafting using the sural nerve are limited, so the muscle-in-vein nerve grafts are the proper solution [8].

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Direct coaptation of restant nerve stumps is a problem often because the surgical intervention is made later and the nerves have the property to retract. In this case a nerve graft is absolutely necessary. The possibilities are allografts, synthetic nerve conduits to conduct axons regeneration or natural conduit filled with muscles, nerve growth factors, fat, collagen.

Every method has advantages and disadvantages, but the cost and site morbidity is often named.

Peripheral nerves are regenerating with approximatively 1 mm per day. This means that a big trauma, with large nerve gaps need more time to heal. The best timing for surgery is 6 months after the trauma but sometimes at this point the resources of direct coaptation are almost inexistent so we need to use other surgical techniques and among them is nerve grafting on different size defects [9].

**Experimental part**

*Material and method*

Animals are anesthetized for surgery using ketamine (100 mg/kg) and xylazine (10 mg/kg) via intramuscular injection. Each animal also receives a subcutaneous injection of meloxicam (10 mg/kg) to minimize post-operative pain and also bupivacaine (1µg/kg) at the site of surgery.

Both hindquarters are carefully shaved using a surgical clippers and depilation is completed with hair removal cream.

Skin is cleansed using sterile cotton tipped applicators and betadine surgical scrub.

Ophthalmic ointment is applied to the eyes using sterile cotton tipped applicators.

The rat is placed on a clean stainless steel plate, under which has been placed on the surgical table. Animal temperature is maintained at 37°C .

All limbs are taped down, with care taken to position the hind limbs symmetrically so that the knee joint makes a right angle with the body. A syringe was placed under the left foot to emphasis the sciatic nerve intraoperatively.

The surgical field is covered with a sterile drape. All instruments are sterilized by autoclave or hot bead sterilization, and we wear a mask, gown, and sterile gloves.

After preparation, a semi-circular incision across midline is made in the skin. The skin is gently dissected from the underlying musculature, and folded over to remain out of the way during the procedure. It is kept moist using applications of 0.1 mL sterile saline during the procedure.

Opening the fascial plane between the gluteus maximus and the anterior head of the biceps femoris reveals the sciatic nerve (fig. 1). For a surgical control, the contralateral sciatic nerve should be exposed and mobilized, but left intact. The gluteal musculature is then re-opposed and sutured using a 6-0 nylon, non-absorbable sutures [10]

The experimental sciatic nerve is then exposed in the same fashion, with retractors in place to ease visualization. The retractors are sterilized prior to use. The sciatic nerve is then gently freed from the surrounding connective tissue using iridectomy scissors.

Using a fine forceps, the nerve is placed on the bottom jaw of a super-fine hemostatic forceps. The three fascicles are sequentially aligned, not on top of each other. The hemostatic forceps have been engraved with a mark at 1.5 mm from their tip. The outermost portion of the sciatic is placed in line with this mark before crush. This ensures a crush of uniform width, and that the nerve does not extend beyond the jaws of the hemostatic forceps when flattened due to the crushing force. If the nerve extends beyond the forceps tip the nerve will only be partially crushed [11].

For our experiment we cut the nerve so we are able to graft it after.

The gluteal musculature is re-opposed and sutured in the same way as the contralateral side.

Finally, the skin incision is closed using 4/0 Nylon sutures. On this model of injury we graft the nerves.

Using the muscle around and a segment from tibial vein we built the muscle-in-vein nerve grafts (Figure 4). On a forceps we glide the entire vein after we washed it in saline solution and heparin. With the tip of the forceps we grab the muscle and glide it into the vein.

The muscle fibers were treated chemically with Triton X-100 and deoxycholate in proportion 2/1 for 5 minutes (5 mL Triton X-100 and 2.5 mL deoxycholate) before clearing it with saline solution and introducing then into the vein.

Triton X-100 and deoxycholate are 2 detergents used in experimental laboratories to create acellular grafts or segments of tissues. The outcomes after using it in nerve grafts were conclusive in comparison with other methods (e.g. freezing-dehydratation method) and this is the reason we used it.

In group 1 we cut the nerve and suture it back after reversing it with 8/0 Premilene under 8x magnification loupes.

In group 2 we built a 3 cm long muscle-in-vein nerve graft from 2 nerve grafts of 1.5 cm centrally sutured.

In group 3 we built a 3 cm long single unit muscle-in-vein nerve graft using ‚window-vein' method.

This nerve graft is 1.5 long for the second group and 3 cm long for the 3rd group but instead of suturing 2 grafts of 1.5 cm centrally, we built a single unit graft using the windows-vein method: in the central part of the vein we made a small perpendicular incision into the vein, then we glide the vein starting from this *window* on the forceps and with the end of the forceps we grab the muscle and pull it until the end. In the same manner we proceed with the second half of the vein. At the end we suture the *window* and our nerve graft is ready for the final suture.

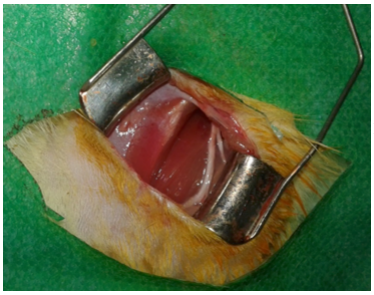

Fig. 1. Actual imagine of the sciatic nerve before neurorraphy. (8mp Samsung camera)

*Post-Operative Care*

Following the procedure, animals are placed on a heating pad at 37°C until they show signs of movement.

They are then moved back to their home cage, where water and food are readily accessible on the floor in the form of Hydrogel and wetted food.

Histology analysis: preservation of the sciatic nerve segments.

Nerve specimens were buffered initially in formaldehyde 37% (12 h). Than we proceed the deparaffinization with xylene I (5 min), xylene II (5 min), 96% ethanol (3-5 min), 70% ethanol (3-5 min) and distilled water (5 min). After this the staining was made with hematoxylin (3-5 min), rinse in water (10-15 min), 70% ethanol (3-5 min) then in eosin (1-3 min). The dehydration was made with 96% ethanol (2 min) and carbon-xylene (3-5 min). The last procedure was clearing with xylene I (5 min) and xylene II (5 min). We mounted with mounting medium and examine the blades is microscope.

Proximal, middle and distal segments of nerve tissue

were examined, revealing a number of myelinated fibers that were counted under the microscope and analyzed using computer imaging.

*The footprint test*

At 12 weeks we performed the footprint test. We marked the back hind of the Wistar rat with methylene blue and put them into a cage of 60x90 cm to walk over a piece of special paper. At the end we measured the size of the back hinds in comparison with each other.

Footprints were evaluated by three parameters: 1) distance from the heel to the third toe, print length; 2) distance from the first to the fifth toes, TS; and 3) distance from the second to the fourth toes, intermediary TS. All three measurements were taken from the experimental (E) and normal (N) sides. Functional recovery was assessed by calculating the Sciatic Functional Index (SFI) value. A value close to 0 indicate a good recovery and a value close to 100 means the opposite [12].

## Results and discussions

A complete investigation of post injury regeneration combines functional, electrophysiological, and morphological assessments. Morphological assessments following regeneration are the ones we are interested in our study.

Analysis of the density of myelinated fibers were comprehensive and showed us a significant difference between group 2 and 3 (P>0.05), meaning group 3 had better results.

Also between group 1 and 2 we have significant difference between the number of myelinated fibers (P>0.05) meaning regeneration was better in group 1 than in group 2.

There no significant difference between the myelinated fibers in group 1 and 3 (p<0.01): this mean that regeneration was made in the same ratio even though the methods were different (table 1).

**Table 1**

THE DIAMETER OF FIBERS

| Diameter of myelinated fibers (µm) | Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| middle | 1.24 | 0.80 | 1.18 |
| margin | 1.22 | 1 | 1.10 |

**Table 2**

THE FOOTPRINT TEST RESULTS

| SFI | Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| Week 12 | 58.1±1.2 | 40.7±1.3 | 53.5±1.8 |

This result are in correlation with the thickness of the perineurium: in group and 3 data are similar but in group 2 the thickness is significantly smaller (table 3).

In group 1 the thickness of the perineurium was significant bigger than group 2 (P>0.05) but no significant difference between group 1 and 3 (P<0.01). This mean that our new method, *window-vein* provides a good regeneration ratio and similar result with direct coaptation in total sciatic nerve trauma.

The density of the fibers in group 1 and 3 was similar but a significant difference was seen between group 1 and 2

**Table 3**

THE THICKNESS OF THE PERINEURIUM

| | Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| Thickness of the perineurium (thickness mµ) | 5.4±2.11 | 4.2±0.85 | 6±2.23 |

(P>0.05) meaning in group 2 the density of the fibers was diminished.

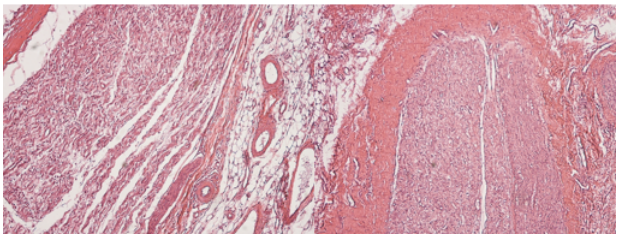

Fig. 2. Group 1. Sciatic nerve suture. Central and lateral aspects. Col HEx20

All the histological result were correlated with functional test.

The footprint test made at 12 weeks revealed a good functional outcome in group 1 and 3 and satisfactory result in group 2. The size of the footprint evaluated by SFI is similar in group 1 and 3 but significant smaller in group 2. This means that the central sutured graft decrease the progress of regeneration and fibrosis and lymphocytes change the course of healing in good conditions (table 2).

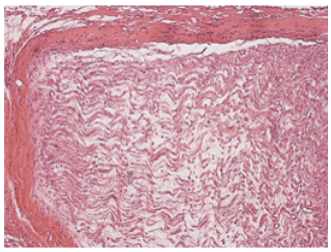

Fig. 3 Group 2. Sciatic nerve grafts using muscle-in-vein graft centrally sutured. (1.5 cmx2). Central and lateral aspect. Col HE x20

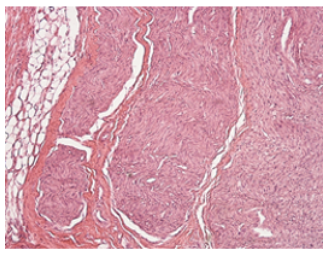

Fig. 4. Group 3. Sciatic nerve graft using muscle-in-vein with *window-vein* method (3 cm). Central and lateral aspects. Col HE x20

Prolonging nerve grafts became an issue in the moment that trauma incidence increased. Since that point, different types of nerve grafts were invented. The synthetic ones have good results nut the cost is ineffective, the allografts are number one choice but the morbidity factor could be a problem at the end. Comparisons can be done with advantages and disadvantages but the conclusion is that we need a nerve graft that is cheap, effective, without adding morbidity and with good functional results.

Our study outcome revealed that muscle-in-vein nerve grafting with ‚window-vein' method is a good possibility of prolonging nerve grafts from 2 cm in live rats to 3-4 cm. This means that in humans the possibility of grafting increases with good functional results.

In group 2 the result were significantly lower than in group 3, by one hand because of the surgical procedure: suturing 2 small muscle-in-vein nerve grafts adds another disturbing factor in axonal sprouting and regeneration, and by other hand muscles sutures slowed down regeneration. Myelin fibers were parallel and thick in group 3 but in group 2 the aspect was poor and chaotic. This can be explained also through the surgical procedure and also by the integrity of the nerve conduit, in this case vein [13,14].

Studies have shown that among other nerve conduits, veins have a better behavior. Their structure nouriches and guide axons to reach the opposite site. Fibers of collagen, elastin, growth factors, endothelin contribute to nerve growing in a proper way [15]. Experimental pain has predefined limits in intensity and duration and it works under various other parameters, as compared to clinical pain [16]. Studies performed in both rodents and human patients stated a possible causal relationship between pain and oxidative stress. In this way, there are some preliminary data which reported that oxidative stress contributes to the reduction in peripheral vascular responses and the maintenance of thermal hyperalgesia in rats[17]. The importance of the oxidative stress led to the idea of using various antioxidants as a possible solution for longer physical training or shorter recovery periods[18].

The humoral immune response disorder is based on a complex interaction between antigen cells and the lymphocytes T and B and a high amount of superoxide radical is also generated by activated neutrophils and macrophages during oxidative burst [19,20].

The morphologic aspect of the nerve specimens in group 3 was comparable with simple suture from group 1. The fibers are dense and thick, Schwann nuclei are in uniformly layer, the perineurium made from vein is thick and uniform, this showed that the axonal sprouting was possible without many disturbance.

In other studies putting the vein inside out has shown to be more effective in nerve regeneration because the exterior membrane of the vein mimicking the perineurium and the axonal sprouting was even faster than in classic muscle-in-vein nerve graft .

Nerve grafting nowadays have the goal to reestablish the function and sensibility of the injured limb as fast as possible. This goal is often hard to reach because nerve trauma have a long delayed timing for surgery. Expecting 6 months before surgery is sometimes not in the advantage of both patient and surgeon. After this period a series of consequences like fibrosis, neuroma, muscle stiffness, paresthesia, vicious positions, make recovery harder and more complicated [21].

The total cost of a synthetic nerve conduit on a 3 cm gap is approximatively 800 euros, but the cost of the same nerve gap reconstructed with muscle-in-vein nerve graft with *window-vein* is zero. All it needs is surgeon work and ability and the same amount of instruments like any other nerve surgery [22]. This advantage might be taken to consideration regarding founds in many hospitals. It would bring more resolved cases and save a lot of found which can be redirected to kinesiotherapy or occupational therapy.

## Conclusions

Muscle-in-vein nerve grafts with *window-vein* method it's an effective method for grafts prolonging. Morphological design revealed a good regeneration and axonal sprouting and also thick perineurium. Further clinical studies are needed to show result in patients and also a longer postoperatively surveillance.

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