Bioactive Nanofiber-based Conduits in a Peripheral Nerve Gap Management- an Animal Model Study.

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Research

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Abstract

Background: Peripheral nerve injuries (PNI) are trauma with severe squeals for patients' quality of life. Currently, the gold standard of nerve reconstruction relies on primary coaptation. However, in case of a nerve gap, a reconstructive material for bridging is needed. Nerve conduits are an increasingly popular solution alternative to autografts or processed nerved allografts. The aim of the study was to examine the efficiency of a novel bioactive nanofiber-based tubular scaffold made of poly (L-lactic acid)-co-poly(ϵ-caprolactone), collagen, polyaniline and enriched with adipose-derived stem cells (ASCs) as a nerve conduit in a rat model.

Methods: Poly (L-lactic acid)-co-poly(ϵ-caprolactone) scaffold was optimized and enriched with collagen (COL) and polyaniline (PANI) to create final (P(LLA-CL)-COL-PANI), tubular-shaped scaffold, manufactured with electrospinning technology. Parallelly, adipose tissue from 10 Lewis rats was harvested for ASC culture. 28 inbred male Lewis rats were divided into four even groups. Each animal underwent sciatic nerve transection and excision of a 10 mm nerve trunk fragment. In group A, nerve gap remained untouched, in B excised trunk was rotated and used as an autograft, in C nerve stumps were secured with P(LLA-CL)-COL-PANI conduit. In the D group gap was reconstructed with P(LLA-CL)-COL-PANI conduit enriched with ASCs. After 6-months of observation rats were sacrificed. Gastrocnemius muscles (operated and non-operated side) and reconstructed sciatic nerves were harvested for analyses. Muscles were weighted and underwent histological analysis. Nerves were processed and stained immunohistochemically with NF-200 to be analyzed with dedicated software for nerve fiber count.

Results: No signs of rejection or excessive fibrosis was noted. Muscle mass ratio was highest in group B (0.77±0.05), then in C (0.74±0,04) and D (0.67± 0.07). Group A showed advanced atrophy of the muscle, each intervention prevented muscle mass decrease ($p<0.0001$), however, ASC addition decreased efficiency vs autograft ($p<0.05$). Nerve fiber count revealed a superior effect in the nerve fiber density observed in the group with the use of conduit vs autograft (D vs B $p<0.0001$, C vs B $p<0.001$). ASC added to the conduit decreased perineurium hyperplasia.

Conclusion:

P(LLA-CL)-COL-PANI conduits with ASC showed promising results in managing nerve gap by decreasing muscle atrophy and providing a favorable environment for peripheral nerve regeneration.

Background

Peripheral nerve injuries (PNI) are one of the most disabling components of traumatic injuries of extremities, especially traumatic hand injuries, and may accompany 2.8–6.1% of cases [1–3]. Sequels of PNI concern sensory or motor malfunction of an injured limb, causing a severe decrease in quality of life.

In the case of nerve shortening after trauma, neuroma resection, or during reconstructive procedure surgeon may encounter a need for management of a gap between nerve stumps. The main goal of the

procedure is to provide a tension-less junction between the ends of an injured nerve since that factor mainly contributes to delayed neuroregeneration [4]. To the date, there are few possible surgical approaches in managing peripheral nerve gaps. Namely, primary microsurgical suturing, use of sutureless techniques (tissue glues), allografts, autografts, and synthetic conduits [5]. Primary suturing remains gold standard, however only in case of tension-free conditions. If the primary approach is impossible, there is a need for creating a bridge between nerve stumps. In the upper extremity, the digital nerve's gap should be treated with processed nerve allograft (PNA), unless distance exceeds 3 cm, then an autograft should be used. Motor or mixed nerves injury of upper extremity always requires the use of an autograft [6]. There are numerous disadvantages to both alternatives. Moreover, autografts require the sacrifice of a donor site of a patient – sural nerve, lateral antebrachial cutaneous nerve or medial antebrachial cutaneous nerve, and cause loss of innervation of a certain area, requires additional cuts and there are a limited number of available sites with acceptable morbidity. Processed allografts do not cause donor site morbidity of a patient but may not be available in numerous countries (legal, ethical, or organizational issues) and may be used in injuries raging 5–50 mm [7, 8]. Conduits emerged as a novel alternative to these solutions and struggle their way to the algorithms in the clinical approach. Currently, conduits are successfully used in digital nerve repair and are an effective alternative to PNA in managing short neural gaps [6]. There are few products approved by the FDA, manufactured from type I collagen, polyglycolic acid, polycaprolactone, polyvinyl alcohol, or porcine small intestine submucosa [9]. Since they are off-the-shelf products, easily stored, accessible, and biocompatible, they pose a serious alternative and direction towards the future solution of nerve repairs.

However, none of these materials exhibit perfect features as a neural bridge for sprouting axons. Thus, exploration of novel material continues, and recently poly(L-lactic acid co-ϵ-caprolactone) based scaffolds are perceived as potential candidates in that application [9].

Conversely, regenerative medicine techniques are intensively explored in the field of neuroregeneration. Harnessing multipotent cells (adipose-derived stem cells- ASC, bone marrow stromal cells - BMSC, mesenchymal stem cells - MSC) for promoting the regeneration of an injured nerve has been widely reported. Thanks to their pleiotropic effect on inflammation, immunomodulation, neurotrophic factors excretion, transdifferentiation into Schwann-like cells they may serve as precious component neuroregenerative therapies [10, 11]. ASC are eagerly investigated in nerve regeneration studies due to their easy harvest and confirmed beneficial influence via exosomes promoting Schwann cells proliferation, migration, and myelination [12]. Yet, alone they are an insufficient weapon in PNI with a long nerve gap. Hundepool et al., in their metanalysis, summarized the supportive role of stem cells in nerve conduits [3].

P(LLA-CL) is a non-toxic biodegradable polyester possessing good mechanical properties and degradation rates and has been successfully utilized in nerve tissue engineering applications [13]. Collagen compound was chosen after a prior optimization study, which revealed superior proneurogenic feature of P(LLA-CL) with collagen [14]. PANI was added because of its electroconductive features, which promote neuroregeneration [15].

The aim of the study was to create novel, yet unknown, poly(L-lactic acid co-ϵ-caprolactone)-derivate conduits and investigate their efficacy in the regeneration of a 10 mm gap in the sciatic nerve of a rat with and without ASC enrichment.

Materials And Methods

Materials

Poly (L-lactic acid)-co-poly(ϵ-caprolactone) [P(LLA-CL)] with a ratio of 70:30 was purchased from Evonik (Germany). Atelocollagen (COL) was purchased from KOKEN (Japan). 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) was obtained from Fluorochem (UK). Polyaniline (PANI) and (1S)-(+)-10-Camphorsulfonic acid (CSA) were purchased from Sigma-Aldrich (USA).

Fabrication of electrospun tubular fibrous scaffolds

A 10 % (w/v) solution of P(LLA-CL) and Col [ratio of 85 to 15] in HFP was mixed with 10 % PANI/CSA [50:50] in HFP solution in the ratio of 7 to 3. The composite solution was then electrospun in the NANON-01 apparatus (MECC, Japan) at a voltage of 15 kV using a needle to tip a working distance of 80 mm and feed rate of 1.0 ml/h. A stainless-steel mandrel with a 2 mm diameter rotating with a speed of 100 rpm was used as a collector. The 21 G needle – spinneret was moving with a speed of 100 mm/sec and the spinneret width was set to 160 mm. The electrospinning process was conducted for 70 minutes. Electrospun tubes after 48 h drying in vacuum drier (25°C, 50 mbar) were extracted from the mandrel.

Characterization of the electrospun scaffolds

The morphology of the obtained fibrous tubes was observed, after sputter coating with gold, using a scanning electron microscope (SEM, PhenomX, The Netherlands) at an accelerating voltage of 10 kV. The diameters of obtained nanofibers were analyzed from the SEM images using Image Analysis Software (ImageJ, National Institute of Health, USA). The average diameter of the fibers was determined by measuring the diameters of 50 randomly selected fibers. The thickness of the walls and the outer diameter of the tubes were measured using a micrometer screw in 5 different places of the tube.

The tensile properties of the electrospun nanofibrous tubes were evaluated using a tensile testing machine Instron 5943 (Instron, USA) at a crosshead speed of 5 mm /min under ambient conditions. The samples for mechanical tests were 20 mm long, and the end of each side of 5mm was attached to the hydraulic clamps.

Hydrophilicty of the tubes was evaluated by the static contact angle measurement method using a contact angle goniometer (OCA 20, Dataphysics, Germany). A volume of 2 µl of DI water was placed on the surface of the samples and 3 seconds after the droplet touched the surface the image was taken.

Measurements were taken at nine different positions and contact angles were calculated using SCA20 software.20.

Harvesting, isolation, differentiation, and seeding of ASCs into the scaffold

10 male Lewis rats were sacrificed and euthanized for fat pad harvest. Fat was harvested in a sterile manner from 4 regions – inguinal, gonadal, pararenal, and nuchal (parascapular). Fat pads were collected in 1 % PBS and immediately transferred on wet ice to the Department of Regenerative Medicine for further ASC isolation. Each fat pad underwent isolation protocol with 0,075 % Type I collagenase consistent with the original protocol proposed by Zhu et al. and previous studies [16, 17]. Isolated stromal vascular fraction (SVF) pellet was dissolved in 2 ml DMEM. Cells underwent immediate and delayed (after 3-5 passage) cytometric analysis (CD29, CD11b, CD90, CD45 and CD34). SVF was also subjected to a differentiation test with alizarin red, Masson's Trichrome, and oil red staining. Finally, MTS and doubling time tests were applied to compare the survival rate of fat harvested from different regions. Ultimately, there were no statistically significant differences in the survival rate of cells and contents in ASCs in any region. Thus, cell culture after 3-5th passage from all regions was used to prepare ASCs stocks for conduits enrichment in further steps.

Animal study design

Animal care and handling were carried out in accordance with the UK's Animals (Scientific Procedures) Act 1986 and associated guidelines, the EU Directive 2010/63/EU for animal experiments, and comply with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The experiments were approved by the Second Local Ethics Committee in Warsaw (Protocol no 72/2015). All surgical procedures were performed using aseptic techniques. Inbred male Lewis rats aged 4-6 months (n = 28), were acclimatized to 12 h light/dark cycle at 19°C, with unlimited water and standard food. The rats weighed 366 to 486 g at the time of surgery.

Twenty eight animals were randomly assigned into 4 even groups, each receiving a unilateral sciatic nerve transection and resection of 10 mm of its trunk and further: A) control, 10 mm gap remained, B) reconstructed with autograft, C) reconstructed with P(LLA-CL)-COL-PANI conduit, D) reconstructed with P(LLA-CL)-COL-PANI conduit and enriched with ASCs (See Figure 1). Animals were anesthetized with Isoflurane. The randomly chosen limb was shaved, sterilized and a 3 cm cut was performed along the dorsal side of a rat's thigh. Gluteal muscles (GM) were divided to expose the sciatic nerve (SN). SN was cleared from the surrounding tissues and measured to obtain a continuous 10 mm trunk to be resected with at least a 5 mm margin from its divisions or branches. The previously marked fragment of SN was resected with a sharp blade. In rats assigned to group A SN was left without coaptation, with both stumps unsecured. Group B was reconstructed with the resected trunk of the sciatic nerve, which was rotated

180° and used as an autograft, finally coaptated with 2 epineural sutures on each side (10/0 Prolene). In group C P(LLA-CL)-COL-PANI conduit was placed between proximal and distal nerve stump, which were further advanced about 2 mm into the lumen of the conduit and secured with 2 epineural sutures on each side (10/0 Prolene). In group D $3x10^6$ ASCs were seeded into a P(LLA-CL)-COL-PANI conduit and incubated for 3 days in 37°C, 5% CO_2 and 95% humidity in separate Petri dishes in DMEM with 10% FBS. Implantation was managed similarly to group C. After conduit securing an additional portion of 0,3ml of cultured ASCs stock containing $1x10^6$ cells was injected with a 32G needle in the lumen of the conduit (in the space between both nerve stumps) (see Figure 2). Afterward, gluteal muscles were secured with two 4/0 Vicryl sutures, and skin was closed with interrupted 4/0 Vicryl sutures. Rats were administered postoperatively with analgesics and remained under strict observation for 6 months. Operated limbs were secured for the prevention of autotomy or self-cannibalism. Scaffolds were sterilized with 25 kGy dose radiation prior to the implantation.

Samples harvest and histological analyses

At 6 months of observation, rats were sacrificed by inhalation using isoflurane overdose (>4% v/v). Samples of conduits (or nerve autografts), ipsilateral and contralateral gastrocnemius muscle were harvested. In group A, discontinuation of SN was confirmed during the harvest. Sciatic nerves were cut out with a 5 mm margin on both proximal and distal sides of conduits, in case of Group B harvest was performed likewise. Additionally, SN and its surroundings were inspected for the presence of inflammation, scarring, fibrosis, or conduit dissolution. Freshly harvested muscles were weighted on a RADWAG AS220 electronic balance (Puszczykowo, Poland) and results were expressed as a ratio of ipsilateral to the contralateral muscle (denervated to innervated). Nerve samples were divided into 3 portions – (A) proximal to the conduit, (B) body of conduit, and (C) distal to the conduit. Further, samples were immediately immersed in a 10% Buffered formalin solution and transferred to the Department of Methodology (Medical University of Warsaw) for further processing. Samples underwent a standard protocol for sample embedding in paraffin. Subsequently, nerves were cut into 4 µm slices and muscles into 10 µm slices. The samples of the muscles were stained with hematoxylin and eosin (HE) with standard protocol and covered with a coverslip. Images of muscle sections were captured under 400x magnification.

Nerve sections in the center of conduit/autograft were stained immunohistochemically with NF-200 primary antibody (N4142, Sigma-Aldrich, Munich, Germany,). Staining was performed according to manufacturer protocol. Samples were counterstained with hematoxylin. Images of stained nerve sections were captured under 400x magnification. Quantitative assessment was performed for sections B. Nerve fiber were identified with the aid of semi-automatic analysis tool – CellProfiler and adjusted protocol reported by Paskal et al. [18]. Nerve fiber density was described as the number of fibers in an mm^2 (axons/mm^2).

Statistical analysis

Data from each study were collected in Excel (Microsoft, Redmond, WA). Kolmogorov-Smirnov test and Shapiro-Wilk test were used to determine the distribution of data. T-test, univariate ANOVA, post-hoc Turkey's tests were used for the analyses accordingly. GraphPad Prism 6 (GraphPad Prism, San Diego, CA) was used for statistical analyses and plots preparation. The threshold of statistical significance is set at $p \leq 0.05$.

Results

Morphology of the electrospun tubes

The average thickness of the wall of the tubes was 272 ± 12 μm and the average outer diameter of the tubes was 2.04 ± 0.04 mm. The inner surface of the tube displayed fibers with an average diameter of 460 ± 143 nm. (see Fig. 3 and Table 1)

Table 1
Parameters of P(LLA-CL)-COL-PANI conduits

Parameter	Value	Units
Wall thickness	272 ± 12	μm
External diameter of a conduit	2.04 ± 0.04	mm
Mean fiber thickness	460 ± 143	nm
Length of a conduit	15	mm
Tensile strength	8.53 ± 1.43	MPa
Elongation to break	332.25 ± 72.11	%
Contact angle	81.23 ± 2.14	o

Animal study

Observation of operated animals lasted 6 months. One rat from group B was euthanatized earlier due to diagnosed actinomyces infection. No wound dehiscence or autotomies were observed. At the time of rats' euthanasia and samples harvest no excessive scarring, fibrosis, or adhesions were observed in any group (see Fig. 4).

Muscular tissue

Gastrocnemius muscles were excised in both hind limbs and weighted. Macroscopically, muscles from the non-operated side were bigger vs ipsilateral to SN lesion (see Fig. 5). It was confirmed with weight analysis which revealed maximal ratio 0.82 in Group B, also mean muscle mass ratio was highest among examined groups (mean ± SD; 0.77 ± 0.05). Insignificantly lower mean muscle mass ratio was observed in

Group C (0.74 ± 0.04). Group D showed the lowest mean muscle mass ratio among groups B, C, and D (0.67 ± 0.07), but statistical significance was observed only when compared to Group B ($p < 0.05$). Each group showed significantly higher mean muscle mass ratio vs Group A ($p < 0.0001$) (see Fig. 6). Macroscopic observations correspond to HE stained images, where the most prevalent influx of inflammatory cells, fibrosis, and muscle atrophy advancement was seen in Group A (see Fig. 7)

Neural tissue

Along with the muscular tissue, fragments of SN were harvested in the final timepoint. NF-200 staining reveals nerve fibers and enables their identification in histological samples. As presented in Fig. 4, in each group with nerve reconstruction nerve continuity was achieved. In Group B autografts presented less compacted appearance vs groups C and D. In group D perineurium were less hyperplastic vs Group C (see Fig. 8). In quantitative analysis, Group D showed the highest nerve fiber density (mean ± SD; 1152 ± 59.16) compared to groups C (1034 ± 67.76) and B (803.6 ± 43.47). The observation was statistically significant ($p < 0.001$ and $p < 0.0001$ respectively) (see Fig. 9).

Discussion

In this study novel P(LLA-CL)-COL-PANI scaffold, with or without ASC enrichment, was analyzed in the context of managing peripheral nerve gaps in a rat model. We aimed to confirm the biocompatibility of the new conduit and compare its efficiency versus gold standard – a nerve autograft.

Both qualitative and quantitative results of muscle tissue analyses indicated that the conduits reconstituted nerve continuity and prevented muscle atrophy which occurred in control group A, in a long, 6-months observation period. However, the addition of ASC to the conduit slightly decreased the beneficial effect vs autograft group. It opposes other studies, where ASC addition either to allografts or conduits was even more profitable for wet muscle mass ratio [19–21]. Yet, statistically significant, the differences between mean values of groups B, C, D remains much lower vs similar studies. Recently, Nakada et al. reported the beneficial effect of enriching a decellularized autografts with ASC, which enabled maintain 60% of wet muscle mass ratio vs autografted group [20]. In our study, conduit or its ASC-enriched version achieved respectively 96% and 87% muscle mass ratio preservation to the autografted group.

Neural tissue analysis revealed convincing superiority of P(LLA-CL)-COL-PANI groups versus autograft. Both groups C and D improved nerve fiber density. In the microscopic analysis, group D showed less pronounced fibrosis of the perineurium than the pure conduit. These observations stay in line with other reports concerning the use of ASC as an additive in nerve regeneration therapies [20, 21]. The similarly beneficial effect of ASC sheet enveloping a decellularized autograft was presented by Nakada et al., however, their solution did not exceed the axonal density of the comparator (1,8 vs 2,29 axons/μm2) as we observed in group D vs B (1152 vs 803 axons/mm2)

ASC implantation in the PNI treatment protocol alters numerous parameters concerning nerve function. They increase nerve fiber or axon count, myelin sheath thickness, improve nerve conduction velocity, sciatic function index (SFI), and muscle mass and prevents them from atrophy and fibrosis [22, 23]. Mainly, undifferentiated to ASCs (uASC) are used in neuroregenerative studies and such cells cannot form myelin itself (in contrast to ASC differentiated to Schwann cells -ASC-SC), but they produce neurite extensions [24]. Nakada along with other authors assumes that the beneficial effect of ASC in conduits relies on the humoral effect of secreted neurotrophic factors [20, 25]. As these studies show, undifferentiated ASCs are not directly involved in myelinization, as they desired in the case of processed allografts or conduits which lack living and interacting cells in their structure. Thus, a combined approach involving ASC-SC padding the conduit lumen with ASC-SC and additional uASC as humoral support should be considered.

The study shows early results of implementing P(LLA-CL)-COL-PANI conduits in peripheral nerve regeneration. To the date, there are no data on P(LLA-CL)- conduits mixed with type I collagen and PANI. However, there are a few recent studies investigating P(LLA-CL) itself or similar derivates in PNI. They proved effective in improving nerve regeneration of a nerve gap [26, 27] or nerve coaptation site wrapping [28]. PCL is also combined with other particles, that may facilitate nerve regeneration, such as laminin [29] graphene [30], carbon nanotubes [31], small porcine intestine submucosa [32]. Thus, P(LLA-CL)-COL-PANI may serve as an effective platform for further enhancements and modifications yet exhibiting considerable proneuroregenerative effect.

The major limitation of the study relies on modest data concerning functional outcomes of nerve regeneration. However, the use of conduits successfully prevented muscle atrophy which is an inevitable consequence of complete peripheral nerve transection and provides objective results unlike, other methods e.g. EMG and ENG [33].

P(LLA-CL)-COL-PANI based scaffold examined in the study showed promising features as a novel alternative in the family of peripheral nerve conduits. Its efficiency in the management of the neural tissue gap is comparable with nerve autografts.

Conclusions

P(LLA-CL)-COL-PANI conduits is biocompatible and provides effective support for axonal regeneration in peripheral nerve gap reconstruction in an animal model. P(LLA-CL)-COL-PANI conduits with ASC enrichment produce a higher density of axons in reconstructed nerve but slightly decrease the wet muscle mass ratio vs P(LLA-CL)-COL-PANI conduit alone. Further histological and functional studies of the conduit are indicated

Abbreviations

Adipose-derived stem cells (ASCs); uASC – undifferentiated ASC, GM – gastrocnemius muscle, P(LLA-CL)-COL-PANI - Poly (L-lactic acid)-co-poly(ϵ-caprolactone) -collagen – polyaniline, SD- standard deviation, SN- sciatic nerve, SC- Schwann cells,

Declarations

Ethics approval and consent to participate:

All animal experiments were approved by the II Local Bioethical Committee in Warsaw (Approval number: 72/2015) and performed according to the Guidelines for the Regulation of Animal Experiments.

Consent for publication:

Not applicable

Availability of data and material:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests:

None

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The sponsor had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors' contributions:

TD designed and conducted the study and performed all surgical procedures on animals, analyzed the data collected during the study and supervised writing of the manuscript, EKG designed the study and designed and manufactured conduits, AZ and KS performed ASCs isolation and differentiation, WP analyzed histopathological samples and wrote the manuscript, PKW supervised histopathological part of the study, WŚ and ZP supervised the entire study.

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Figures

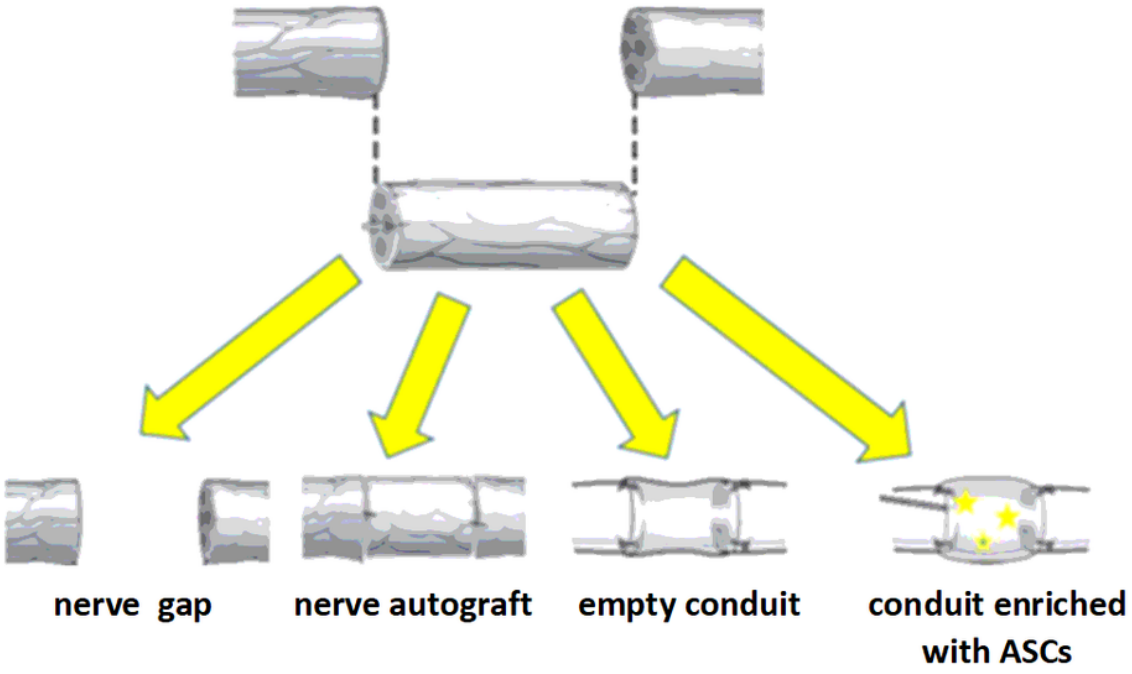

Figure 1

Diagram represents four interventions (groups) to the sciatic nerve. After sciatic nerve transection and excision of 10 mm trunk: A) nerve gap remained, B) excised trunk was rotated 180° and used as an autograft C) P(LLA-CL)-COL-PANI conduit was inserted in the gap D) P(LLA-CL)-COL-PANI enriched with ASCs was inserted in the gap.

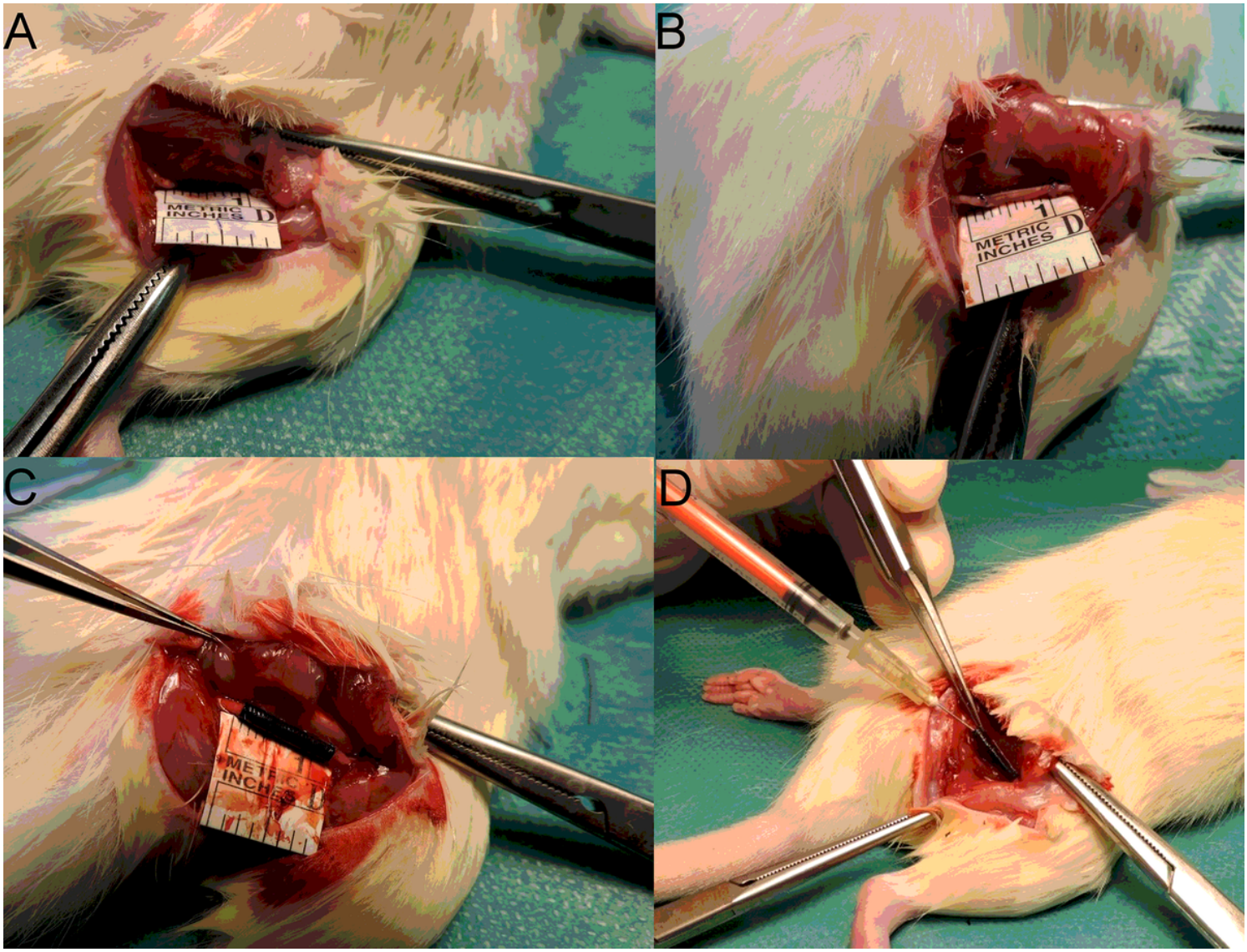

Figure 2

Intraoperative photograph of each experimental group. A) after excision of 10mm trunk of the sciatic nerve 10mm gap remained and the length was confirmed with a millimeter ruler. B) excised trunk was rotated and sutured to the nerve stumps with two 10/0 Prolene epineural sutures. C) P(LLA-CL)-COL-PANI conduit filled the gap, nerve stumps were placed 2 mm into the lumen of the conduit and secured with two 10/ Prolene epineural sutures. D) In group D after ASCs seeded conduit implantation injection of 1x106 ASCs was made into the lumen of the conduit.

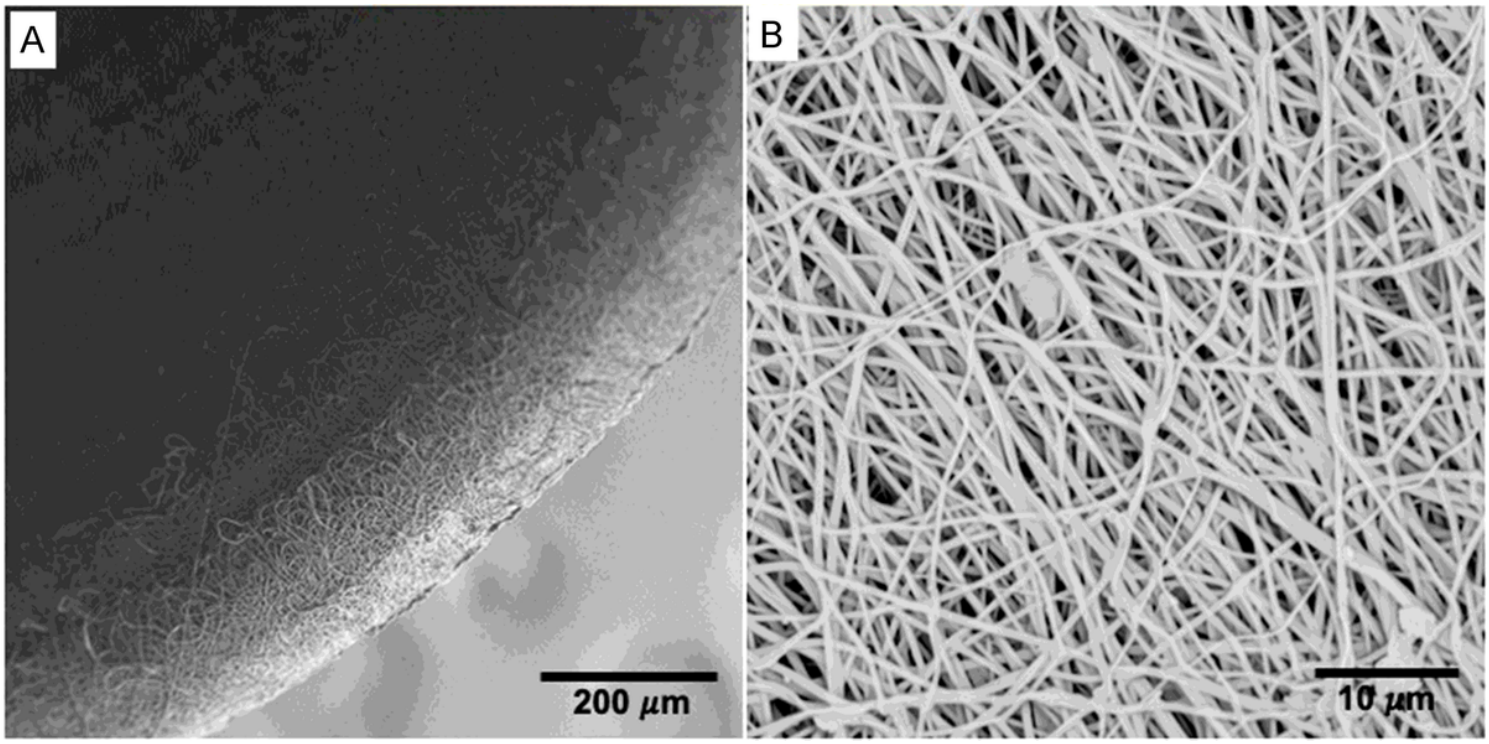

Figure 3

SEM images of electrospun P(LLA-CL)-COL-PANI conduits. A) View into the inner part of a tubular scaffold. B) Magnification on the structure of the scaffold.

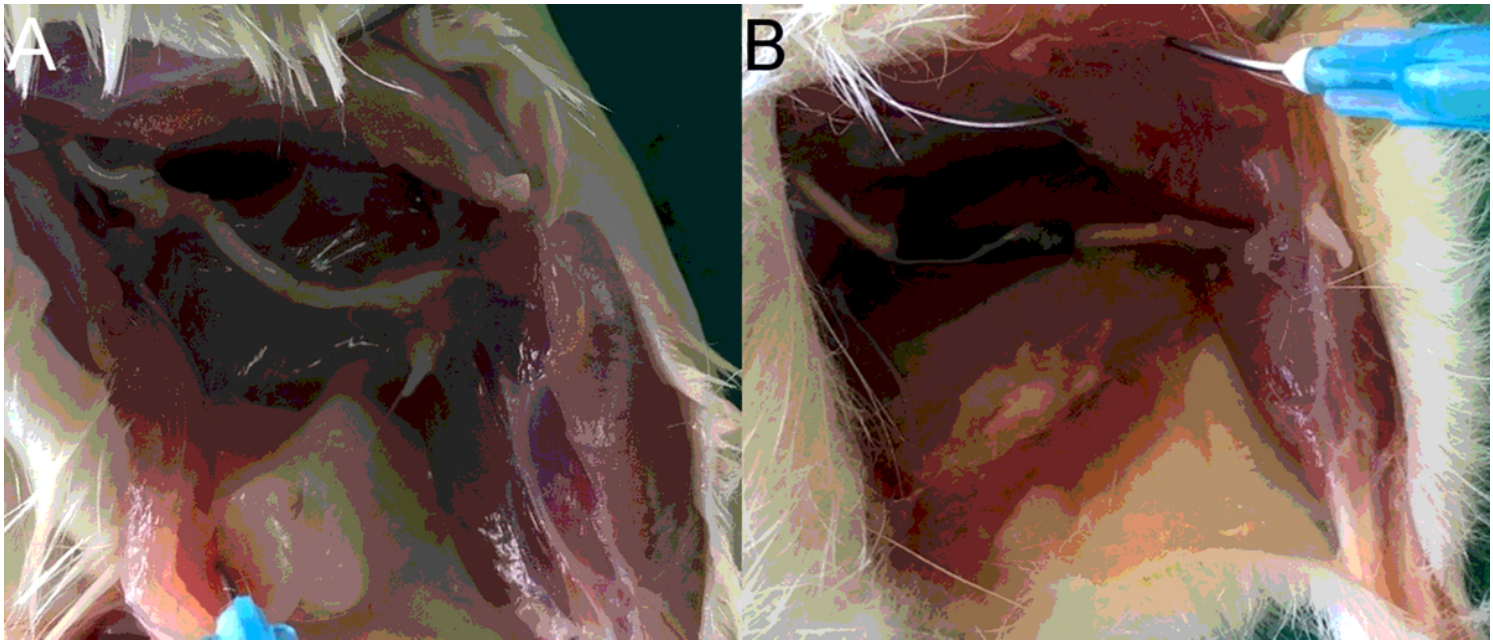

Figure 4

6 months after interventions sciatic nerve were excised for further analyses. A) Group B – sciatic nerve reconstructed with autograft after 6 months, B) Groups C and D – sciatic nerve reconstructed with conduits after 6 months.

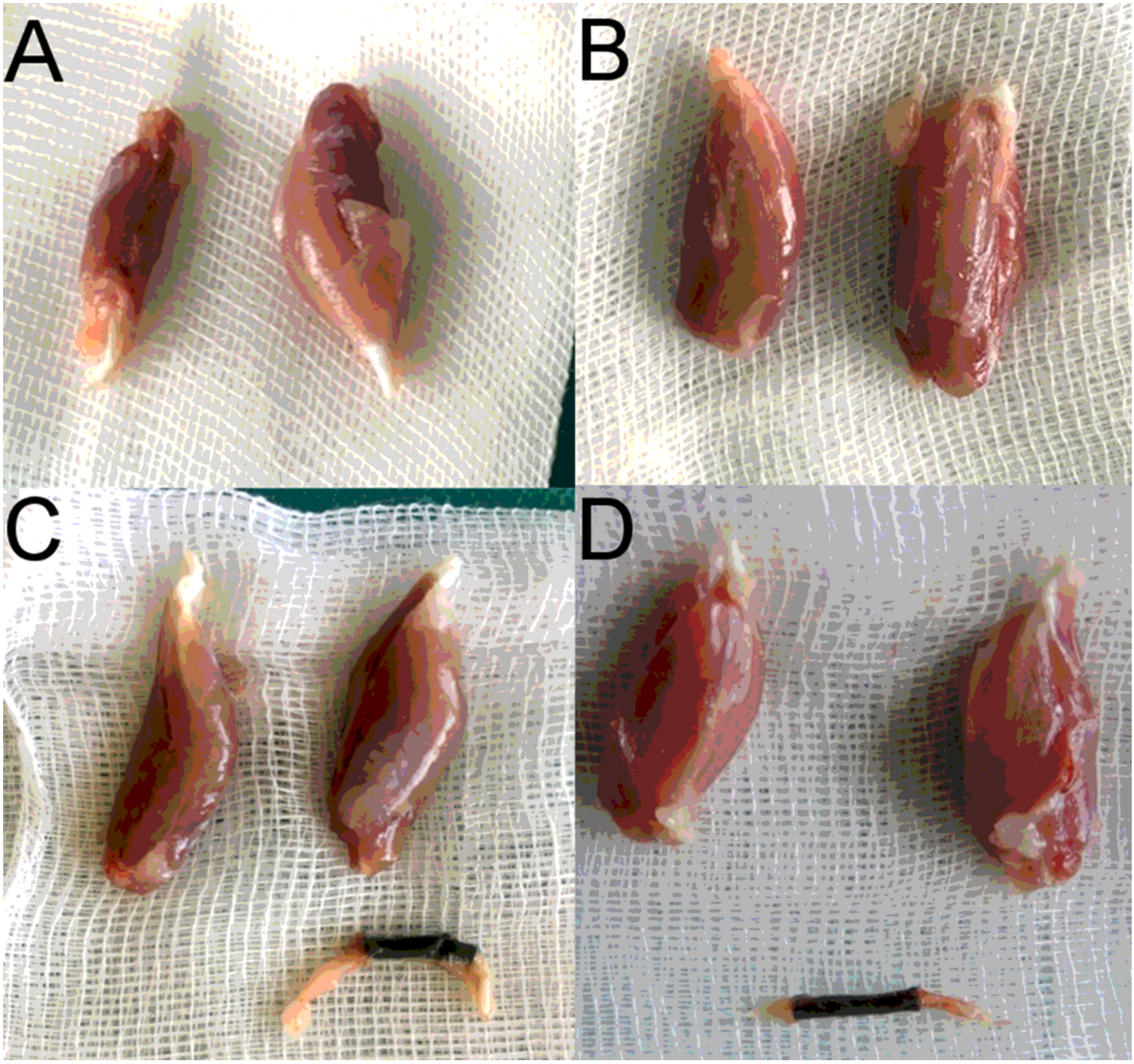

Figure 5

Postoperative images of gastrocnemius muscle excised after 6 months from interventions. On the left side – muscle from an operated limb, on the right side – muscle from the side after reconstructive procedure A) control group – nerve gap remained, the muscle was innervated and decreased its size vs unoperated limb on the right. B) autograft - muscle size is comparable. C) P(LLA-CL)-COL-PANI conduit integrated into the sciatic nerve (bottom), it substantially preserved gastrocnemius size (left). D) P(LLA-CL)-COL-PANI conduit was enriched with ASC cells prevented the muscle from atrophy (left vs right)

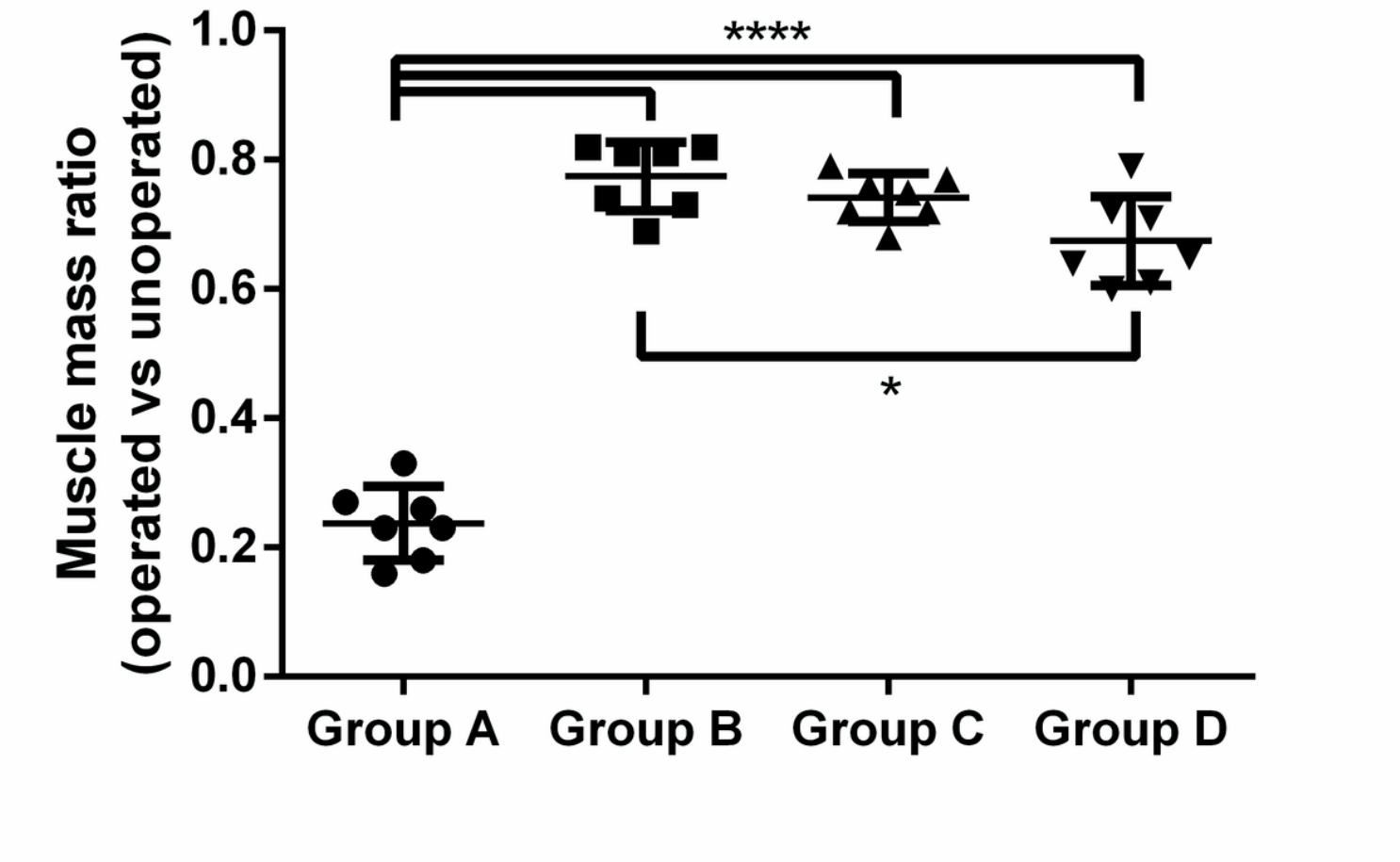

Figure 6

Diagram represents muscle mass ratio (operated vs unoperated limb) among examined groups – A) nerve gap, B) autograft C) P(LLA-CL)-COL-PANI conduit, D) P(LLA-CL)-COL-PANI with ASCs. Mean±SD, *- $p<0.05$, **** - $p<0.0001$

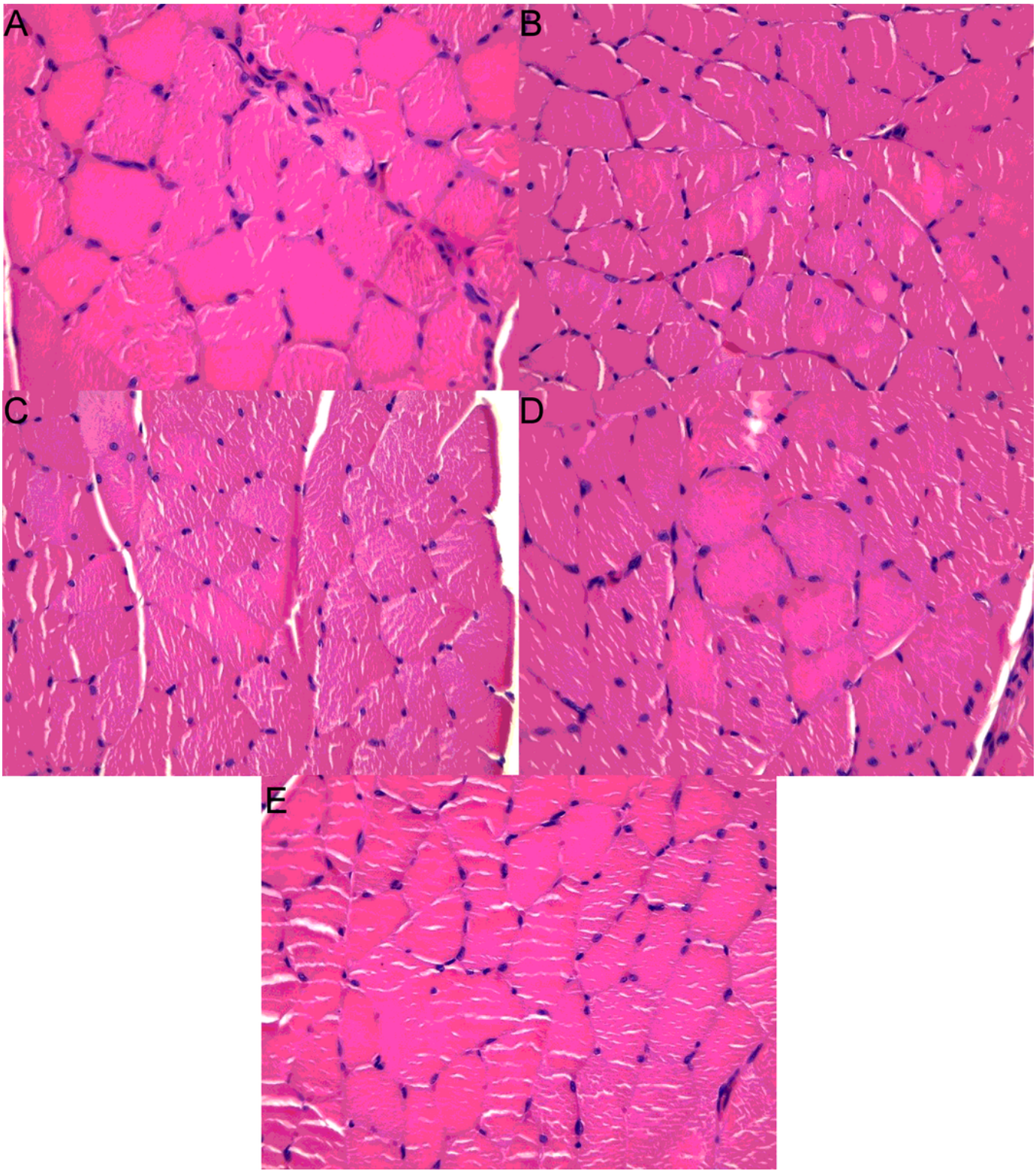

Figure 7

HE images of gastrocnemius muscle sections from the operated limb (A-D) and from random unoperated limb (E). A) in nerve gap group muscle fiber size is decreased; a higher number of nuclei was observed along with the increased prevalence of connective tissue between muscle fibers and inclusion bodies B) autograft group - slightly increased number of inclusion bodies and increased number of nuclei and inclusion bodies. C) P(LLA-CL)-COL-PANI conduit group – comparing to E) slightly higher number of

nuclei and connective tissue fibers were noted. D) P(LLA-CL)-COL-PANI conduit with ASCs group – a higher number of inclusion bodies was noted. E) histologic image of a correct, unaffected muscle.

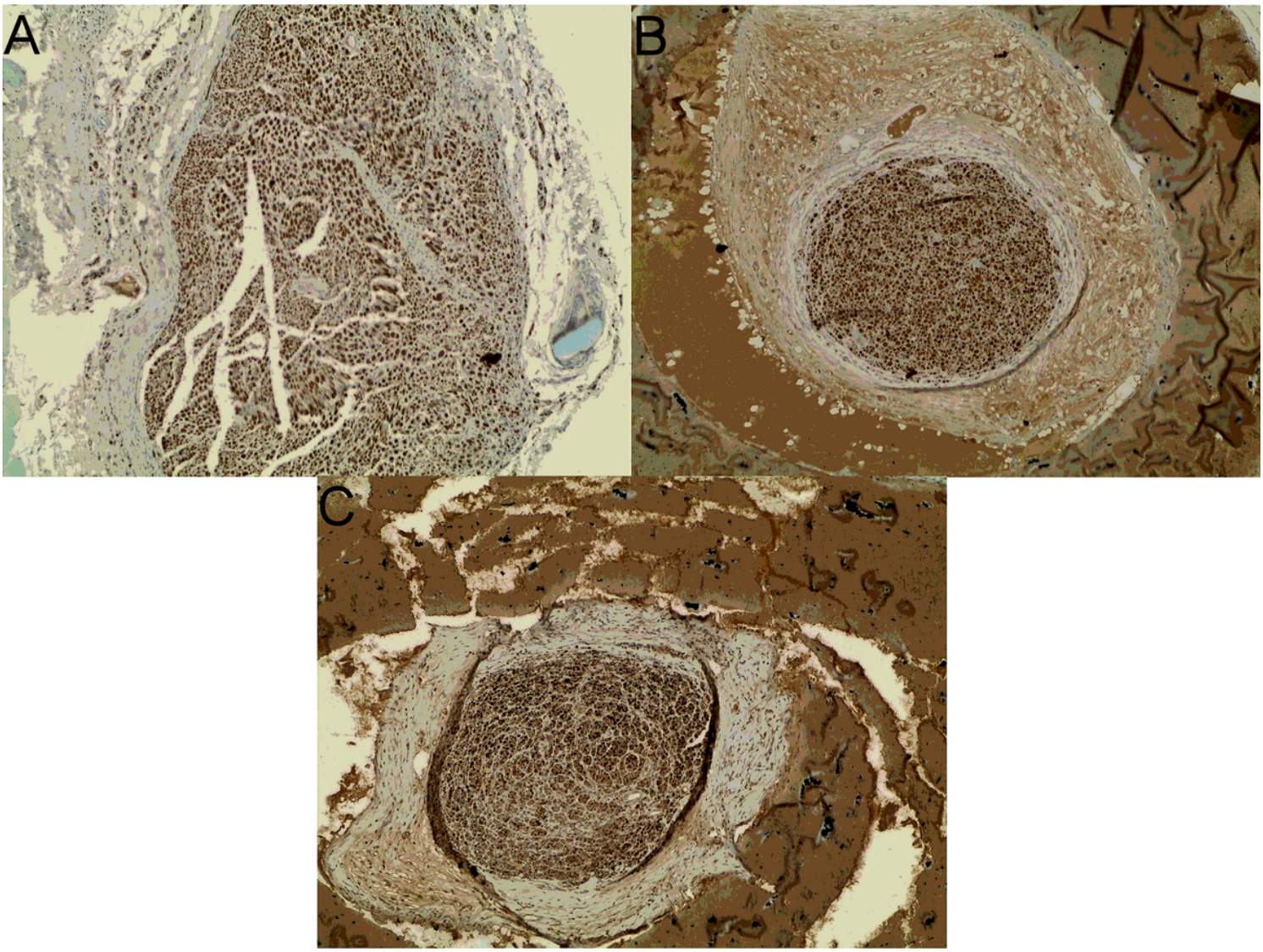

Figure 8

NF-200 immunohistochemistry images of nerve sections in the center of conduit/autograft. A) autograft group, gentle inclusion of fibrotic tissue with the graft were noted, a high number of well-developed nerve fiber were present with medium-sized myelin sheaths, regular morphology of perineurium B) P(LLA-CL)-COL-PANI conduit group – fiber are densely encapsulated within the lumen of the conduit, perineurium is significantly developed and often exceeds 50% of the area of the conduit lumen, yet without signs of fibrosis, scarring or compression within nerve fiber tissues. The outer layer consists of partially dissolved conduit C) P(LLA-CL)-COL-PANI conduit with ASC group – density of nerve fiber within the epineurium sheath is high, without scarring of fibrosis. Perineurium is thick, but to a lesser extent vs conduit alone, it does not fully fill the lumen of a conduit. The outer layer consists of a partially dissolved conduit

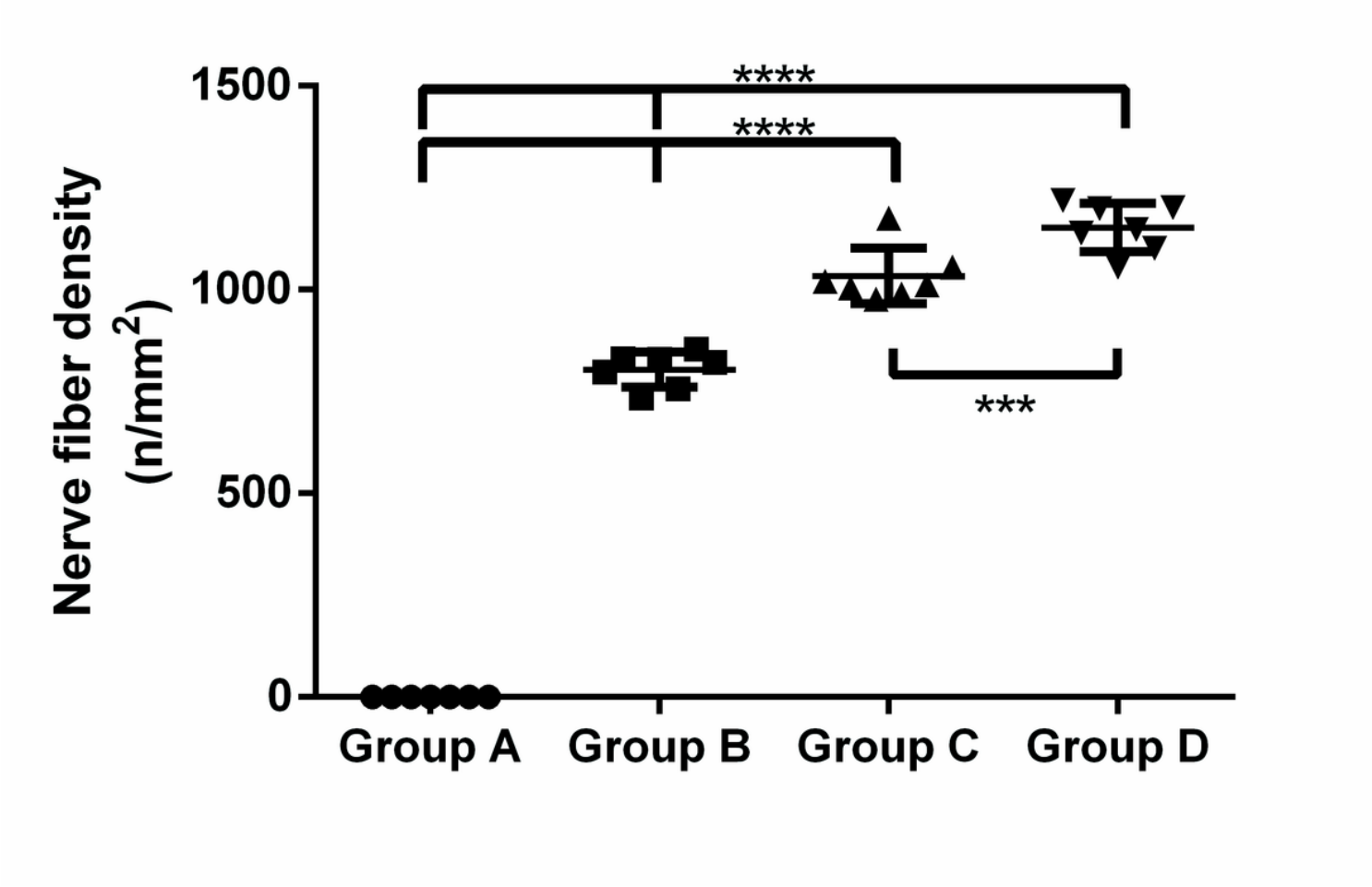

Figure 9

Diagram represents nerve fiber density calculated from NF200 stained images among examined groups – A) nerve gap B) autograft C) P(LLA-CL)-COL-PANI conduit, D) P(LLA-CL)-COL-PANI with ASCs. Mean±SD, ***-p<0.001, **** - p<0.0001