Hypoxic pre-conditioned adipose-derived stem/progenitor cells embedded in fibrin conduits promote peripheral nerve regeneration in a sciatic nerve graft model

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Abstract
Recent results emphasize the supportive effects of adipose-derived multipotent stem/progenitor cells (ADSPCs) in peripheral nerve recovery. Cultivation under hypoxia is considered to enhance the release of the regenerative potential of ADSPCs. This study aimed to examine whether peripheral nerve regeneration in a rat model of autologous sciatic nerve graft benefits from an additional custom-made fibrin conduit seeded with hypoxic pre-conditioned (2% oxygen for 72 hours) autologous ADSPCs (n = 9). This treatment mode was compared with three others: fibrin conduit seeded with ADSPCs cultivated under normoxic conditions (n = 9); non-cell-carrying conduit (n = 9); and nerve autograft only (n = 9). A 16-week follow-up included functional testing (sciatic functional index and static sciatic index) as well as postmortem muscle mass analyses and morphometric nerve evaluations (histology, g-ratio, axon density, and diameter). At 8 weeks, the hypoxic pre-conditioned group achieved significantly higher sciatic functional index/static sciatic index scores than the other three groups, indicating faster functional regeneration. Furthermore, histologic evaluation showed significantly increased axon outgrowth/branching, axon density, remyelination, and a reduced relative connective tissue area. Hypoxic pre-conditioned ADSPCs seeded in fibrin conduits are a promising adjunct to current nerve autografts. Further studies are needed to understand the underlying cellular mechanism and to investigate a potential application in clinical practice.

Key Words: adipose-derived progenitor cells; adipose-derived multipotent stem/progenitor cell; autologous nerve graft; fibrin conduit; hypoxia; hypoxic preconditioning; nerve defect; nerve tissue engineering; peripheral nerve regeneration; regenerative medicine

Introduction
Severe injuries, especially to patients’ extremities, are often associated with acute peripheral nerve trauma. Incomplete axonal regeneration can greatly compromise function and quality of life and is associated with a high financial burden on the healthcare system (Raza et al., 2020). Whenever feasible, an appropriate tension-free end-to-end coaptation of the stumps is recommended (Diao and Vannueny, 2000). In peripheral nerve defects of a critical size, autografting is still considered the clinical gold standard, but donor site morbidity remains a significant drawback (Kuffer and Foy, 2020). Nerve conduits (e.g., of bioresorbable materials or autologous vessel grafts) as guiding structures for axonal outgrowth and targeting have been evaluated and found to be a satisfactory treatment option for defects < 20 mm. Current research in peripheral nerve regeneration focuses on cell-based therapies to further axonal regeneration (Brooks et al., 2012; Daly et al., 2012; Braga Silva et al., 2017; Bingham et al., 2019). Recent results from experimental models emphasize the supportive effects of easily harvested adipose-derived multipotent stem/progenitor cells (ADSPCs) in peripheral nerve recovery and regeneration (Reichenberger et al., 2016; Sowa et al., 2016; Masgutov et al., 2019). The therapeutic potential of mesenchymal stromal cells, such as ADSPCs, can be modulated by various techniques of cellular pre-conditioning (Ferreira et al., 2018). These include treatment of stem cells with pharmacologic agents, growth factors or hormones, or incubation in a 3-dimensional culture and can be modulated by various techniques of cellular pre-conditioning (Ferreira et al., 2018). There is evidence that hypoxic cultivation of mesenchymal stem cells can lead to upregulation of the transcription factor hypoxia-inducible factor 1 alpha, which subsequently results in enhanced protection against oxidative stress and increases vascularization by elevated concentrations of vascular endothelial growth factor and angiogens, both supportive factors in the regenerative process (Ahlawala and Tarrnawski, 2012; Kiani et al., 2013). Furthermore, hypoxic pre-conditioning was shown to increase the stemness of hMSC by upregulating important pluripotency genes, including KLF4, NANOG, and OCT4 (Wei et al., 2017).

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To release the potential of a cell-based regenerative approach at the site of injury, a scaffold matrix is required, as direct injection of cells often results in poor engraftment. The ideal scaffold must meet a long list of requirements, including biocompatibility and biodegradability, promotion of cell adhesion and proliferation, and adequate cell nutrition. As a scaffold carrier for ADSPCs, the clinically approved natural biomaterial fibrin shines showing promise in vitro characteristics (Krug et al., 2019), such as homogeneous cell distribution within the fibrin matrix, high biocompatibility, high degradability, and proven cell viability within the matrix over the long term, as well as metabolic, migrating and remodeling cell activities. Additionally, fibrin conduits themselves are capable of guiding axon regrowth and reducing neuroma formation, consequently benefitting nerve recovery (Issacs, 2013).

In this in vivo animal study, we examined whether nerve regeneration after surgical paralysis or intramuscularly seeded nerve defects on an autologous fibrin conduit can significantly be improved by the addition of a fibrin conduit seeded with undifferentiated hypoxic pre-conditioned autologous ADSPCs.

**Methods**

**Study design**
The rats were handled and housed according to the federal and institutional guidelines for the care and use of laboratory animals approved by the government of Upper Bavaria on May 23, 2014 (approval No. 55.2-1.54-2532-177-13), as follows: controlled temperature of 20–22°C, 12-hour light/dark cycle, moderate room ventilation, ice-water bath to initiate hypothermia, intramuscular injection of 0.02 mg/kg fentanyl (Janssen, Neuss, Germany), 1.0 mg/kg midozolam (Solvay, Brussels, Belgium), and 0.2 mg/kg medetomidine (Orion, Espoo, Finland). Anesthesia was postoperatively antagonized with 0.03 mg/kg naloxone (Bristol-Myers Squibb, New York, USA) and 0.2 mg/kg atipamezole (cp-pharma, Burgdorf, Germany). The animals were positioned on a 37°C heating plate (Tempcontrol 37 – 2 digital, PeCon GmbH, Erbach, Germany) to prevent hypothermia.

The animals were randomly assigned to four groups (n = 9). Three different therapeutic regimens were compared with the current clinical gold standard, which served as a control (group AT):

- **AT group**: Nerve autograft plus fibrin conduit seeded with hypoxic preconditioned autologous ADSPCs;
- **ATCN group**: Nerve autograft plus fibrin conduit seeded with autologous ADSPCs cultured under normoxic conditions;
- **ATC group**: Nerve autograft plus fibrin conduit;
- **AT group**: Nerve autograft.

A 16-week postoperative follow-up of lower extremity functional improvement was conducted before sacrificing the animals for histological assessment.

**Surgical intervention**
The autologous nerve graft model, a well-established peripheral nerve defect model in rats, has been shown to be reliable in vivo (Saller et al., 2018). All surgical procedures were performed under sterile conditions. Pre-operative anesthesia was initiated by intramuscular injection of 0.02 mg/kg fentanyl (Janssen, Neuss, Germany), 1.0 mg/kg midozolam (Ratiopharm GmbH, Ulm, Germany) and 0.2 mg/kg medetomidin (Orion, Espoo, Finnland). Anesthesia was postoperatively antagonized with 0.03 mg/kg naloxone (Bristol-Myers Squibb, New York, USA) and 0.2 mg/kg atipamezole (Roche, Basel, Switzerland) and 1.0 mg/kg atipamezole (cp-pharma, Burgdorf, Germany). The animals were positioned on a 37°C heating plate (Tempcontrol 37 – 2 digital, PeCon GmbH, Erbach, Germany) to prevent hypothermia.

Three days before the operation, inguinal fat pads were resected bilaterally to harvest ADSPCs. Wounds were closed with interrupted absorbable sutures (Vicryl 4-0, Ethicon, Norderstedt, Germany).

To induce an artificial nerve defect of critical length (20 mm or longer in rats), we followed a previously published surgical protocol (Saller et al., 2018). In brief, animals were placed on a warming device and the right hind limb was shaved and sterilized. The skin was incised and, after a blunt intermuscular split of the superficial gluteal and biceps femoris muscles, the sciatic nerve was exposed. Between its proximal origin close to the spine and its division into its branches (common peroneal nerve and tibial nerve), a 20-mm-long segment was resected and reverse-positioned into the neural gap to imitate the mismatch when bridging a critically sized nerve defect by an autologous nerve transplant. Proximal and distal microsurgical epineural coaptations were carried out with non-absorbable thread (Ethilon 9-0, Ethicon, Norderstedt, Germany). After the nerve coaptation, the cell-seeded or non-cell-seeded fibrin conduit (see below) was placed around the nerve transplant. The conduit was immersed in standard culture medium and mixed in a 1:10 ratio with thrombin. The cell-seeded conduits were applied during surgery as described above.

To determine lower extremity functional recovery, a walking track analysis of the sciatic function index (SFI) and a footprint image analysis of the static sciatic index (SSI) were conducted preoperatively and on postoperative weeks 4, 8, 12, and 16. Both indices are reliable methods to evaluate functional peripheral nerve recovery after injury and repair to the sciatic nerve of rats (de Medinaceli et al., 1982; Bervar, 2000). The hind feet of the animals were marked with dark ink, and the animals had to pass through a 110-cm-long tunnel. A cover at the end of the tunnel served as a shelter, thus motivating them to traverse the walking track deterministically produce accurate footprints. To document the SFI postoperative walking, an array of right-angled, high-resolution 300 dpi gray scale images of acrylic glass with a transparent floor plate. The parameters for the indices were measured manually with the open source software ImageJ (version 1.51d; NIH, Bethesda, MD, USA; Schneider et al., 2012).

The three variables required to calculate the SFI are the print length, the toe spread between the first and fifth toes, and the intermediate toe spread between the second and fourth toes. The SFI is defined by the toe spread and the intermediate toe spread (de Medinaceli et al., 1982; Bervar, 2000). The print length is not relevant to this non-dynamic index. For both indices, scores of nearly 100 display complete impairment of the sciatic nerve, while scores around 0 indicate normal function.

**Muscle mass analysis**

Peripheral nerve injuries can affect the motor innervation of target muscles. As a consequence, results in muscular atrophy, nerve regeneration can be evaluated by the analysis of the degree of atrophy, resulting in an inverse correlation of muscle atrophy and mass values. Bilateral gastrocnemius muscles that are innervated by the tibial nerve, which branches from the sciatic nerve, were harvested after animals were sacrificed by CO₂ chamber euthanasia (100% CO₂, fill rate of 50–70% CO₂ of the chamber volume per minute) at 16 weeks. The wet masses of the muscles of the impaired and contralateral limbs were determined.

**Histological assessment**

Sciatic nerve segments of about 30 mm length, including the autograft zone, 2 mm segments from distal and proximal sciatic nerve segments, were harvested for histological evaluation of axon recovery. The contralateral sciatic nerve was collected as a control. Samples were fixed overnight in 4% paraformaldehyde in PBS (Carl Roth, Karlsruhe, Germany) and embedded in paraffin (Paraplast Plus, Leica Biosystems, Nussloch, Germany) and then cut into 4-µm semi thin cross-sections with a sliding microtome (Leica SM2000R, Leica Biosystems, Nussloch Germany). After deparaffination with xylol (Merck, Darmstadt, Germany) and rehydration in a decreasing ethanol row, sections were stained with 1% toluidine blue (Merck) solution for 2 minutes, washed in distilled water, and mounted. Fully automated microscopic (400x magnification; Axioliner Observer, Zeiss, Jena, Germany) images were taken and stored. The computer-assisted assessment of the number of myelinated and unmyelinated nerve fibers was determined.

**Autologous ADSPC isolation and culture**

After surgical harvest, the inguinal fat tissue was washed with phosphate-buffered saline (PBS), minced, and enzymatically (Matrase, Sigma, Germany) digested. The cell suspension was suspended in the connective tissue was removed and the available cell suspension (ARC-10–Processing Unit, InGenomer) according to the manufacturer’s protocol (Krug et al., 2016). Isolated cells were resuspended in standard culture medium (89% DMEM, 10% heat inactivated fetal calf serum (HyClone, South Logan, UT, USA), 1% Penicillin/Streptomycin, 1% NEURAL REGENERATION RESEARCH | Vol 18 | No. 3 | March 2023 | 653

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tissue, the mean axon diameter, and the g-ratio (axon myelination)—i.e., the ratio of axon diameter to myelinated fiber diameter—were determined. As scar formation in the regeneration site affects axon regeneration in the central nervous system (Anderson et al., 2016) and similar mechanisms might be involved in the regeneration of peripheral nerves, we quantified the relative connective tissue area in the proximal and distal parts of the defect. As the electrophysiologic properties of regenerating axons strongly depend on remyelination (Gillespie and Stein, 1983) and axon diameter, both were quantified in an unbiased and automatic manner.

**Statistical analysis**

All analyses were performed by investigators who were not blinded to experimental condition. For all statistical comparisons, a Kruskal Wallis test was calculated with GraphPad Prism (version 8, GraphPad Software Inc., La Jolla, CA, USA). A P-value of < 0.05 was considered significant. All data are represented as box plots (median, 25%/75% quartiles, and range).

**Results**

All 36 animals were included in the analysis, all without pathologic findings regarding their general condition for the entire study period.

Conduits with hypoxic pre-treated cells lead to a faster functional recovery

Four weeks after surgery, animals of all groups showed no significant intergroup difference in gait recovery (SFI; Figure 1A). Applying a fibrin conduit only and a fibrin conduit seeded with cells resulted in a significantly improved SFI compared with that in the AT group (P < 0.01 and P < 0.001; Figure 1A’). At 8 weeks, animals in the ATCH group had a significantly increased SFI values compared with those of the ATCN (P < 0.05) and ATC groups (P < 0.01; Figure 1B). In addition, the SSI values in the ATCH group at 8 weeks were significantly improved over the other groups (P < 0.05 or P < 0.001) (Figure 1B’). The SSI values in the ATCN group were the same as the ATC group.

At 12 weeks, the SFI values in the ATCN group were significantly improved over those in the ATC group (P < 0.05; Figure 1C and D). Although not statistically significant, the trend in the ATCH group was toward a better performance than in the other groups (Figure 1C’, C’, D and D’ and Additional Figure 1).

Reduced scar formation and increased axon density in rats with cell seeded conduits

In all groups, no coaptation failure or neuroma formation was found macroscopically when the nerve segments were harvested at 16 weeks. Descriptive evaluation of toluidine blue stained semi-thin nerve sections showed that the number of axons, axon size and myelination appeared to be reduced in animals in the AT (Figure 2A), ATC (Figure 2C) and ATCH (Figure 2B) groups compared with the healthy contralateral side. Treatment regimens in the ATCH and ATCN groups did not lead to a larger axon diameter in the distal end (Figure 3D), but to a reduction in the loss of myelin in the distal defect end compared with the findings in the AT and ATC groups (Figure 3E). In addition, plotting the axon diameter versus the g-ratio indicated that, compared with a healthy sciatic nerve, the application of cell seeded fibrin conduits (ATCH and ATCN groups) led to an axon diameter/g-ratio that was closer to healthy than in the ATC and AT groups (Figure 3F).

**Figure 1 | Hypoxia pretreated AD5PSC loaded fibrin conduits lead to a faster functional peripheral nerve regeneration.**

The sciatic functional index (SFI) and static sciatic index (SSI) on postoperative weeks 4, 8, 12, and 16. All values are presented as box plots (median, 25%/75% quartiles, and range). The g-ratio indicated that, compared with a healthy sciatic nerve, the application of cell seeded fibrin conduits (ATCH and ATCN groups) led to an axon diameter/g-ratio that was closer to healthy than in the ATC and AT groups (Figure 3F).

**Figure 2 | Descriptive nerve morphology.**

Examples of toluidine blue stained semi-thin nerve sections, which reveal that the number of axons, axon size and myelination seem to be increased in adipose derived multipotent stem/progenitor cells treated groups and proximal regions of animals treated with hypoxic cultured adipose derived multipotent stem/progenitor cells seem more homogeneous. Boxes represent the magnifications in A–E and B’–E’ (scale bars: 200 µm; inserts: 50 µm). AT: Autologous transplant; ATC: autologous transplant plus conduit; CS: contralateral side (native sciatic nerve).
In summary, slightly less muscle atrophy was seen when hypoxic pre treated cells seeded in a fibrin conduit were applied. In the distal defect region, the relative connective tissue area was significantly decreased in the ATCH group compared with the AT and ATC groups. An increase in axon outgrowth/branching was found in the ATCH group.

Discussion

Minor nerve defects can successfully be repaired with allografts or artificial nerve tubes. As an alternative to autografts, these approaches eliminate donor site morbidity (Brooks et al., 2012). However, neural tubes cannot bridge a defect of more than 20-30 mm. In these cases, autografts remain the treatment of choice. Thus, successful recovery of motor and sensory function after peripheral nerve repair of discontinuities of a few millimeters strictly depends on the degree of temporal coaptation. However, additives to the coaptation site have been shown to promote the complex processes of nerve regeneration (Diao and Vannuyen, 2000; Jiang et al., 2017). The model used in this study is a true critical-size defect model, which assesses that the defect is sealed in the sciatic nerve, and does not heal on its own. In consequence, anything applied into the gap to heal the defect is the actual cause of any resulting recovery of function. In the animal model used here, the criterion of a critical nerve gap (20 mm or longer) is met. This is a crucial methodologic/technical prerequisite because rats are endowed with an inherent capability to regenerate short nerve gaps (Daly et al., 2012). A special feature of the animal model is that, whenever a nerve autograft is needed, the excised sciatic nerve is itself used. To mimic better the conditions of the clinical problem of nerve gap regeneration, the excised nerve was flipped 180° before coaptation. In the present study, we explored possibilities to improve nerve regeneration after autologous nerve grafting (Jiang et al., 2017; Kuffer and Foy, 2020).

Adding a sole fibrin conduit to the autograft yields a faster and better functional recovery. This is in line with earlier findings, which indicate that fibrin conduits aid nerve growth regardless of the used oxygen preconditioning of the nerve gaps. In the distal stump of the nerve, senescence from denervation neurotrophism—consequently benefitting nerve recovery (Issacs, 2013). Our novel fibrin conduit design and fabrication provide stable and reproducible tissue regeneration, with conformation and cell distribution, which was shown in an earlier study (Krug et al., 2019). Furthermore, the interaction (e.g., degradation rate of fibrin, ADSPCs-release rate) of the fibrin glue and differently cultivated ADSPCs was investigated in vivo. This was performed in this in vivo study. The creation of the conduit itself is not very time-consuming, which may be of economic relevance for a potential clinical application.

Recent in vitro experiments on ADSPCs embedded in a fibrin matrix revealed that the metabolic activity of ADSPCs is significantly higher in a hypoxic setting than in a normoxic setting (Krug et al., 2019). In our study, hypoxic pre-conditioning of transplanted cells significantly accelerated the recovery of the rats’ physical performance when compared with that in rats treated with a fibrin conduit or a conduit loaded with normoxic cultivated cells. Muscle atrophy was the least in the hypoxia group; however, the differences were not significant and can therefore only be seen as a trend. The functional findings also indicate a significant advantage of hypoxic pre-conditioning by own tissue-cultivated and normoxic cultivation of the initial phase of nerve regeneration (see SFI/SIS at 8 weeks). The quicker the autograft is revascularized, the earlier the neural recovery begins (Kuffer and Foy, 2020).

The sooner and quicker the axons extend distally, the fewer Schwann cells of the host and nerve cells are affected by the loss of axons ("Wallerian degeneration"); thus, less of their capacity to proliferate and release neurotrophic factors is lost (Kuffer and Foy, 2020). These more favorable findings of the hypoxic pre-conditioned group could be explained by higher cell proliferation activity, cell survival, and better response to environmental factors and cellular stresses, and the idea that hypoxia enhances the proliferation of ADSPCs and stimulates the regenerative potential of ADSPCs by the upregulation of growth factors (Chung et al., 2009; Kiani et al., 2013). The capacity to adopt a proangiogenic phenotype by hypoxia (Thangarajah et al., 2009) may have the potential to enhance the ability of ADSPCs to promote neovascularization, which is proven to support regenerative processes. Fu et al. (2018) published in vivo data on the use of ADSPCs in a rat model, which is like cells and Schwann-like cell proliferation. While (trans-)differentiation of ADSPCs towards neural tissue types has been speculated to explain the effect of ADSPCs, there is some evidence that paracrine secretion of neurotrophic, regenerative and angiogenic factors (such as brain-derived neurotrophic factor, nerve growth factor, neurotrophin-3, glial cell line-derived neurotrophic factor and vascular endothelial growth factor) is of higher relevance to peripheral nerve regeneration (Kingham et al., 2007; Erba et al., 2010; Solheim et al., 2012; Faroni et al., 2013). Interestingly, however, other authors have conjectured active contribution of ADSPCs in regenerative processes by detection of vital ADSPCs at the nerve coaptation site (Reichenberger et al., 2016). Further trials are crucial to evaluate the exact in vivo behavior and mechanisms of ADSPCs at the site of nerve regeneration.

Scarf formation is considered an inhibiting factor for nerve regeneration (Thangarajah et al., 2009). We found a remarkable relative connective tissue portion, which can be considered as less scarring, in the distal nerve stumps of hypoxic-cell-treated animals. The higher the connective tissue percentage, the worse the regeneration and the more likely neurotrophism (Thangarajah et al., 2004). The more axons grow through the distal stump of the nerve, the more favorable the target re-innervation and subsequent neurological restitution. In our work, unbiased and automatic quantification of histological cross-sections revealed trends of increased axon outgrowth in cell loaded groups and a g-ratio of 0.75% in the hypoxic pre-conditioned group, which could be critical for finding better outcomes and may require further investigation. Severe peripheral nerve injuries can lead to a steeper increase in axon transplantation coverage and axonal density over time in the nerve microenvironment. In this regard, the in vitro hypoxic treatment may fail to exert a sustainable effect on ADSPCs. The in vivo performance and fate of ADSPCs and whether ADSPCs can maintain their hypoxic state are as yet unclear. These are key considerations regarding hypoxic pre-conditioning and whether it is worth the effort. It may explain why only a modest difference was detected between the ATCH and ATCN groups. In vitro data regarding these aspects is already available. Additional monitoring of the in vivo processes inside the conduit, such as its biodegradation, as well as an understanding of the cellular and molecular behaviour of the ADSPCs in the fibrin matrix in vivo, would have added value to the study and will be included in future investigations.

Regarding possible transfer of our findings to clinical application, a closer look at the aspect of hypoxia is imperative. Hypoxia in the context of cell culture is defined as oxygen concentration from 0% to 20% (Graf and Schachtrup, 2010; Etehadifar et al., 2015). It is relevant to bear in mind that oxygen concentrations of this range represent "in situ normoxia" for most human tissues, including fat tissue (Etehadifar et al., 2015). Normal oxygen concentration (atmospheric pressure of 21% O2), as routinely applied for standard cell culture, is higher than the oxygen concentration in fat tissue given in physiological in vivo conditions. Time-consuming pretreatment and expansion (even outside of the operating room) may not be necessary in the clinical application of this therapy.

In accordance with current literature (Mazini et al., 2019), our observations are encouraging. ADSPCs in custom made fibrin scaffolds appear to be a promising strategy for enhancement of peripheral nerve recovery. Another noteworthy aspect for clinical application is that ADSPCs provide a sufficient cell number for regeneration in humans. It is easy to handle and may be used to avoid the limitation of extensive donor site morbidity. The focus of this study was the use of pre-conditioned ADSPCs. However, attention must be paid to the latest findings on the role of exosomes in peripheral nerve regeneration (Ching and Kingham, 2015; Liu et al., 2020). Recently published studies indicate that the use of ADSPC-derived exosomes, engineered to encapsulate NT-3 for example, are a promising approach to enhance peripheral nerve regeneration (Li et al., 2018; Yang et al., 2021).

Further investigations comparing the effects of an exosome based therapy and autologous pre-conditioned ADSPCs, or even a combination, might be of interest.

Nevertheless, ethical restrictions in engineering such exosomes or the processing of ADSPCs before implantation have to be considered critically, as in most countries “modifications such as centrifuging and accumulating cell populations do require the permission of an ethical committee and/or regulatory agencies” (Saller et al., 2018). Another very considerable aspect regarding clinical transfer of our approach is patient safety with regard to drug compatibility. The fibrin glue used is clinically approved, biocompatible, preserves high cell viability, and is already utilized in a broad range of clinical applications (Krug et al., 2019).

One limitation of the study is that only endpoint histomorphological data are provided. Moreover, immunofluorescence staining of axons and myelin for 200-kDa neurofilament protein and myelin basic protein and detection of the related indicators of nerve regeneration from the transcriptional or translational level would have added further value and should be integrated into future studies.

In summary, adding hypoxic pre-conditioned ADSPCs embedded in a custom-made fibrin conduit to nerve autografting in a sciatic nerve defect of critical size was revealed to be beneficial for remyelination, axon outgrowth/branching, avoidance of muscle atrophy, and motor function. However, physiological function and histology were not entirely restored within 16 weeks’ follow-up. To ensure a proper transfer of the benefits to clinical practice, further studies will have to focus on the underlying mechanisms of how ADSPCs in a fibrin matrix promote peripheral nerve regeneration after autologous nerve grafting. The approval for clinical use of exosomes derived ADSPCs, clinically approved fibrin glue may already be used to improve nerve regeneration after autologous nerve grafting.

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Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary files.

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Additional file: Additional Figure 1: Examples of footprints of rats in the ATCH and ATCH groups on postoperative weeks 4 and 16.

References

Ahluwalia A, Tarnawski AS (2011) Critical role of hypoxia sensor--HIF-Jalphap in VEGF gene activation. Implications for angiogenesis and tissue injury healing. Curr Med Chem 18:99-107.

Anderson MA, Burda JE, Ren Y, Ao Y, O'Shea TM, Wiberg M, Terenghi G (2007) Mesenchymal stromal cell secretome: influencing therapeutic potential by cellular communication. Stem Cell Res Ther 12:442.

Braga Silva J, Marchese GM, Cauduro CG, Debiasi M (2017) Nerve conduits for treating peripheral nerve regeneration: bridging the peripheral nerve gap and enhancing axon regeneration. Stem Cells Dev 26:189-196.

Deming TJ, Sofroniew MV (2016) Astrocyte scar formation aids central nervous system axon regeneration. Nature 532:195-200.

Ivanovic Z (2009) Hypoxia or in situ normoxia: The stem cell paradigm. J Cell Physiol 219:271-275.

Jiang L, Jones S, Jia X (2017) Stem cell transplantation for peripheral nerve regeneration: current options and opportunities. Int J Mol Sci 18:94.

Kamal, Kazemi A, Halabian R,Mohammadipour M, Jahan-Najafabadi A, Roudkani MH (2013) HIF-Jalphap confers resistance to induced stress in bone marrow-derived mesenchymal stem cells. Arch Med Res 44:185-193.

Kingham PJ, Kalberratten MF, Mahay D, Armstrong SJ, Wiberg M, Terenghi G (2007) Adipose-derived stem cells promote peripheral nerve regeneration in vivo without inducing ectopic mass formation. Stem Cells Dev 16:1-11.

Krug C, Beer A, Schrott PH, Holzbach T, Guenther E, Müller R, Volkmann E (2019) Fibrin glue displays promising in vitro characteristics as a potential carrier of adipose progenitor cells for tissue regeneration. J Tissue Eng Regen Med 13:359-368.

Kurowski R, Tishler VS, Weinberger RS, Agostini NM, Trepel M, Stedman R, Wolf K (2019) Adipose-derived mesenchymal stem cells for use in peripheral nerve regeneration. J Cell Physiol 234:10823-10834.

Lee HU, Kim M, Kim T, Mino K, Park YN, Lee EK, Kim JS, Park JS, Park KJ, Kim SI, Lee HJ (2020) Stem cell therapy to promote limb function recovery in peripheral nerve damage in a rat model: Experimental research. Ann Plast Surg (London) 40:1-20.

Braga Silva J, Marchese GM, Cauduro CG, Debiasi M (2017) Nerve conduits for treating peripheral nerve injuries: A systematic literature review. Hand Surg Rehabil 36:71-85.

Brooks DN, Weber RV, Chao JD, Rinker BD, Zoldos J, Robichaux MR, Ruggeri SB, Anderson MA, Burda JE, Ren Y, Ao Y, O'Shea TM, Wiberg M, Terenghi G (2007) Mesenchymal stromal cell secretome: influencing therapeutic potential by cellular communication. Stem Cell Res Ther 12:442.

Margutov R, Masgutova G, Mullahmetova A, Zhuravleva M, Shulman A, Rogozhin A, Syromiatnikova V, Andreuza D, Zinvalia A, Idrisova K, Allguerci C, Kiyasov A, Rizvanov A (2019) Adipose-derived mesenchymal stem cells applied in fibrin glue stimulate peripheral nerve regeneration. Regen Med 13:359-368.

Mazini L, Rochette L, Amine M, Malka G (2019) Regenerative capacity of adipose derived stem cells (ADSCs), comparison with mesenchymal stem cells (MSCs). Int J Mol Sci 20:2523.

Mitha R, Grochmal J (2019) Surgery for nerve injury: current and future perspectives. J Neurosurg 130:675-685.

Raja R, Riazi A, Anjum R, Shakeel NUA (2020) Repair strategies for injured peripheral nerve: Review. Life Sci 243:117308.

Reichenberger MA, Mueller W, Hartmann J, Diehm J, Lass U, Koellensperger E, Leimer R, Tatsch K, Midha R, Grochmal J (2019) Surgery for nerve injury: current and future perspectives. J Neurosurg 130:675-685.

Sowa Y, Imura T, Numajiri K, Nishino K, Fukushima S (2012) Adipose-derived stem cells produce factors enhancing peripheral nerve regeneration: influence of age and anatomic site of origin. Stem Cells Dev 21:1852-1862.

Sowa Y, Ishida K, Nishino K, Tabata Y, Mazda O (2016) Adipose-derived stem cells promote peripheral nerve regeneration in vivo without differentiation into schwann-like lineage. Plast Reconstr Surg 137:318-330e.

Tannemaat MR, Boer GJ, Eggers R, Malejsky MJ, Verhaegen J (2009) From microsurgery to nanosurgery: how viral vectors may help repair the peripheral nerve. Prog Brain Res 167:67-78.

Wei ZZ, Zhu YB, Zhang JY, McCrary MR, Wang S, Zhang YB, Yu SP, Wei L (2017) Priming of mesenchymal stem cells in a rat model of cavernous nerve injury. Andrology 6:927-935.

Wu SC, Yang CY, Lin YD, Liu YF, Xie Z, English AW, Li QF, Lin HD (2020) Effect of exosomes from adipose-derived stem cells on the apoptosis of Schwann cells in peripheral nerve injury. CNS Neurosci Ther 26:189-196.

Zeng Q, Ding Y, Liu D, Zhang Y, Liu H (2017) Adipose-derived mesenchymal stem cells promote peripheral nerve regeneration: a novel model and validation. Stem Cells Dev 26:1832-1843.

Zhu Y, Guo A, Fu B, Zhao S, Teng H, Sun Y, Zhang Q, Zhao J, Yang F, Shi F, Guo Y, Song H, Sun Q, Xu Z, Guan R (2018) Exosomes derived from mesenchymal stem cells exert therapeutic effect in a rat model of cavernous nerve injury. Andrology 6:927-935.

Zhu Y, Guo A, Fu B, Zhao S, Teng H, Sun Y, Zhang Q, Zhao J, Yang F, Shi F, Guo Y, Song H, Sun Q, Xu Z, Guan R (2018) Exosomes derived from mesenchymal stem cells exert therapeutic effect in a rat model of cavernous nerve injury. Andrology 6:927-935.
Additional Figure 1 Examples of footprints of rats in the ATCN and ATCH groups on postoperative weeks 4 and 16.