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Extracorporeal shockwave treatment: A novel tool to improve Schwann cell isolation and culture

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Abstract

Background aims. As new approaches for peripheral nerve regeneration are sought, there is an increasing demand for native Schwann cells for *in vitro* testing and/or reimplantation. Extracorporeal shockwave treatment (ESWT) is an emergent technology in the field of regenerative medicine that has also recently been shown to improve peripheral nerve regeneration. *Methods.* In this study, we elucidate the effects of ESWT on Schwann cell isolation and culture. Rat sciatic nerves were dissected and treated with ESWT, and Schwann cells were isolated and cultured for 15 passages. *Results.* Single treatment of the whole nerve *ex vivo* led to significantly increased extracellular adenosinetriphosphate as an immediate consequence, and subsequently a number of effects on the culture were observed, starting with a significantly increased Schwann cell yield after isolation. In the ESWT group, the quality of culture, reflected in consistently higher purity (S100b, morphology), proliferation rate (5-bromo-2-deoxyuridine, population doublings per passage) and expression of regenerative phenotype-associated markers (P75, glial fibrillary acidic protein, c-Jun), was significantly improved. In contrast, the control group exhibited progressively senescent behavior, reflected in a decrease of proliferation, loss of specific markers and increase in $P16^{INK4A}$ expression. *Conclusions.* ESWT has beneficial effects on Schwann cell isolation and culture.

Key Words: *extracorporeal shockwave treatment, peripheral nerve regeneration, Schwann cells*

Background

Peripheral nerve lesions occur with an incidence of approximately 300 000 cases annually in Europe, representing a frequent cause of hospitalization and displaying a major burden to patients and social health care [1].

Although the peripheral nerve system has a remarkable regenerative potential, regeneration over nerve gaps or over long distances (e.g., after proximal lesions) presents several difficulties. In this regard, nerve autografts are the gold standard to treat peripheral nerve injuries with tissue loss but often do not result in a satisfactory outcome [2]. In particular, long-distance gaps or severe injuries affecting several nerves push autografting to its limits regarding the availability of donor material. Alternatives to facilitate nerve regeneration, such as artificial nerve guidance tubes or other types of scaffolds or application of neurotrophic substances, are sought. Some of these approaches are currently used in clinical nerve repair, although there is an ongoing debate concerning their appropriate use, effectiveness and side effects [3,4]. One of the major reasons for the unsatisfactory outcome after repair of long-distance gaps is the limited proliferative capacity of Schwann cells [5]. Schwann cells play a key role in peripheral nerve regeneration: they participate in the removal of myelin and axonal remnants, start proliferation and align to build the so-called bands of Büngner [6]. After the axon has

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elongated along the bands of Büngner, the Schwann cells start to remyelinate the newly formed axon to complete the regenerative process.

A novel strategy to improve the functional outcome of peripheral nerve regeneration is the therapy of injured nerves with extracorporeal shockwave treatment (ESWT). ESWT has its origin in the field of urology, in which it is used to destroy kidney stones [7], but it has also been proven to be an effective therapeutic tool in the field of regenerative medicine. In preclinical and clinical trials, beneficial effects have been reported in treatment of various medical indications such as non-union fractures [8–10], ischemia-induced tissue necrosis [11], or chronic wounds [12,13]. The shockwave generated is a sonic pulse and is characterized by an initial rise, reaching a positive peak of up to 100 MPa within 10 ns, followed by a negative amplitude of up to −10 MPa and a total life cycle of less than 10 μs. Biological responses are thought to be triggered by the high initial pressure, followed by a tensile force and the resulting mechanical stimulation [14].

Recently, Hausner et al. [15] showed a novel approach of accelerating regeneration after peripheral nerve injury, bridged with an autologous nerve graft. After dissecting and bridging the sciatic nerve of a Sprague-Dawley rat, extracorporeal shockwaves were applied at the site of injury. Six weeks after surgery, animals of the ESWT group exhibited a significantly improved functional recovery relative to controls. On the basis of this study, we investigated *in vitro* Schwann cell behavior after ESWT treatment with focus on their regenerative capacity.

Methods

Shockwave treatment of nerve tissue and Schwann cell isolation

All animals were euthanized according to established protocols, which were approved by the City Government of Vienna, Austria, in accordance with the Austrian Law and Guide for the Care and Use of Laboratory Animals as defined by the National Institutes of Health. Animals and treatment/control groups were randomly chosen and analyzed without pre- or post-selection of the respective nerves or cultures.

For *ex vivo* shockwave treatment an unfocused electro-hydraulic device was used (Dermagold 100, MTS Medical. The applicator was attached to a water bath as described in other studies [16–18], ensuring direct contact to the pre-warmed (37°C) water, allowing reproducible physical propagation and application of shockwaves *in vitro*. Sciatic nerves of adult male Sprague-Dawley rats were dissected, and each nerve was transferred into a 15-mL conical centrifuge tube (PAA Laboratories) containing phosphate buffered saline (PBS; PAA Laboratories) pre-chilled on ice. Nerves were kept on ice until further use but not longer than 1 h. For ESWT application, tubes were placed 5 cm in front of the applicator inside the water container. Subsequently, unfocused shockwaves were applied using the parameters chosen according to previous experiments [15] to maximize the effect of the ESWT treatment, while minimizing possible negative effects: 300 pulses at an energy level of 0.10 mJ/mm^2 with a frequency of 3 Hz. The corresponding second nerve from the same animal served as control and was placed in a water bath (37°C) for the time of treatment to avoid the creation of artifacts due to different sample treatments.

After ESWT treatment, Schwann cells were isolated from the treated and non-treated sciatic nerve tissues according to a method adapted from Kaewkhaw et al. [19]. Briefly, the epineurium was removed and nerves were weighed on a fine scale to assess nerve wet weight (Sartorius). Nerves were subsequently strained and minced. Nerve fragments were incubated with 0.05% collagenase (Sigma-Aldrich) for 1 h at 37°C and then filtered through a 40-μm cell strainer and centrifuged at 400*g* for 6 min. After washing the cell pellet with Dulbecco's Modified Eagle Medium (DMEM; PAA Laboratories) containing 10% fetal calf serum (FCS; PAA Laboratories), the pellet was resuspended in DMEM-D-valine (PAA Laboratories), supplemented with 10% FCS, 2 mmol/L L-glutamine (PAA, Austria), 1% antibiotics (PAA Laboratories), N_2 supplement (Invitrogen), 10 μg/mL bovine pituitary extract (Sigma-Aldrich), 5 μmol/L forskolin (Sigma-Aldrich). This medium is subsequently referred to as "Schwann cell medium." Cell suspension was seeded on six-well plates (PAA Laboratories) coated with poly-L-lysin (Sigma-Aldrich) and laminin (Sigma-Aldrich).

Cell culture and experimental setup

Cells were subcultured for the first time after 19 days, to establish a proliferative phenotype and keep them in a proliferative state. Schwann cell medium was added on day 5 after isolation (1 mL) and was partially (50%) changed on days 9, 13 and 17. Subsequent splitting of cells was performed for 15 passages as follows: cells were detached with a cell scraper, centrifuged at 1200 rpm for 5 min and seeded at a density of 4×10^4 cells/cm^2 on plates previously coated with poly-L-lysin. Residual cells were used for flow cytometric analysis, 5-bromo-2-deoxyuridine uptake (BrdU) assay and protein isolation. Medium was partially (50%) changed every third day, and cells were split every sixth day.

Evaluation of cell yield

To evaluate cell yield, representative phase contrast pictures were taken from each culture using a Leica DMI6000B microscope (Leica), and cells were counted using a Bio-Rad TC20 automated cell counter (Bio-Rad Laboratories). Non-viable cells were identified and excluded by trypan blue staining. Cell count was normalized to 10-mg nerve wet weight assessed before isolation.

Proliferation assay

Proliferation assay using a BrdU (Cell Proliferation ELISA Assay Kit; Roche Diagnostics) for quantitative evaluation of Schwann cell proliferation was performed according to manufacturer's instructions. Briefly, poly-L-lysine coated 96-well plates were seeded with cells at a density of 2.5×10^4 cells/cm^2. After 48 h, medium was changed to Schwann cell medium containing 100 µmol/L BrdU, and cells were incubated for 24 h at standard cell culture conditions (37°C and 5% CO_2). The culture plates were fixated with FixDenat solution and subsequently incubated with anti-BrdU POD antibody solution for 60 min at room temperature. After washing the plate with PBS, tetramethyl benzidine was added for 30 min as a substrate. The reaction was stopped with 1 mol/L H_2SO_4 and absorption was measured at 450 nm with 690 nm as reference wavelength on an automatic microplate reader (Tecan Sunrise).

Flow cytometric analysis

Purity of the Schwann cell cultures was evaluated with flow cytometry for common Schwann cell markers: anti-S100b (rabbit polyclonal; Dako), anti-P75 NGFR (goat polyclonal; Santa Cruz Biotechnology) and anti-P0 (rabbit polyclonal; Santa Cruz Biotechnology). Antibodies were labeled with allophycocyanin (Lynx Rapid Conjugation Kit, ABD Serotec). For analysis, cells were detached with a cell scraper and incubated with the antibodies (1:200) on ice and in the dark for 20 min. Cell pellets were washed twice and resuspended in 200 µL PBS. Flow cytometric analysis (10 000 events) was performed with a BD FACS Canto II (Becton Dickinson), and data were evaluated with Flowjo Version 8.8 (Tree Star).

Immunoblotting

Total protein of cells was extracted using Trizol (peqGold TriFast, Peqlab) according to manufacturer's instructions. Briefly, proteins were precipitated from organic phase with ethanol and pelleted by centrifugation (12 000*g*, 10 min, 4°C). Protein pellet was washed three times with 0.3 mol/L guanidine hydrochloride (Sigma-Aldrich) in 95% ethanol and once with 100% ethanol (Merck), with each washing step followed by centrifugation (7500*g*, 5 min, 4°C). Supernatants were discarded and dry protein pellets solubilized in 1% SDS (Sigma-Aldrich) in analytical grade water.

Equal amounts of protein (up to 3 µg/lane; one donor per gel: passage 2, passage 7, passage 15) were separated on a 12% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. Membranes were blocked with 5% skim milk in Tris buffered saline containing 1% Triton-X100 (TBS-T; Sigma-Aldrich) for 120 min and incubated with primary antibodies S100b (Dako), c-Jun (Abcam), glial fibrillary acidic protein (GFAP; Bioss USA), $P16^{INK4A}$ (Abcam), α-tubulin (Calbiochem) diluted in 5% bovine serum albumin (Sigma-Aldrich) in TBS-T at 4°C on a roll mixer for 12 h. Membranes were washed twice with TBS-T and incubated with the secondary antibody in 5% milk-TBS-T. Signals were detected using an Odyssey Fc infrared imaging system (LI-COR Biosciences). After membranes were incubated in 1 × NewBlot IR Stripping Buffer (LI-COR Biosciences) on a shaker at room temperature for 5 min and washed three times in PBS, membranes were reprobed with total antibodies. Ratio of analyzed protein to housekeeping gene α-tubulin was densitometrically analysed using Image Studio Version 5.0.21 (LI-COR Biosciences).

Activation switch

In passages 4, 9 and 15, the activation status and the capacity to switch activation status (proliferating to pro-myelinating) were assessed. Cells were split (2×10^4 cells/cm^2, 24 h adherence time) in two groups: one was cultured in Schwann cell medium and the other in basic medium (DMEM-D-valine; PAA Laboratories), supplemented with 10% FCS, 2 mmol/L L-glutamine, 1% antibiotics) without supplements favoring the proliferating or the pro-myelinating status, respectively. Proliferation behaviour (BrdU enzyme-linked immunosorbent assay) and marker expression (flow cytometry) were assessed 5 days after medium switch.

Adenosinetriphosphate release and lactate dehydrogenase release

The amount of adenosinetriphosphate (ATP) released into the supernatant from nerve tissue treated with ESWT was determined using the CellTiter-Glo assay (Promega). Sciatic nerves were dissected and kept in PBS on ice until further treatment. After removing the epineurium and teasing of the nerve fibers with a mounted needle (15 times in the direction of the fiber), remaining nerve tissue was placed in 500 µL DMEM. Shockwave treatment was performed at 37°C

and with following parameters: 300 pulses with 3 Hz and 0.03 mJ/mm^2, 0.10 mJ/mm^2 or 0.19 mJ/mm^2. The control group was placed in a water bath (37°C). Nerve tissue was incubated for 5 min on ice and subsequently centrifuged at 1500 rpm for 5 min at 4°C. Supernatant was transferred to a micronic tube (150 µL) for lactate dehydrogenase (LDH) measurement (Cobas C111; Roche Diagnostics) and a 96-well plate (triplicate, 100 µL) for ATP measurement. An equal amount of CellTiter-Glo reagent was added, the plate was horizontally shaken for 2 min, and after incubation for 10 min at room temperature, the resulting luminescence was measured. ATP standards were used for calibration of the measured luminescence. After sampling of the initial supernatant, fresh, ice-chilled DMEM was added on nerve tissue and was incubated on ice for another 30 min. ATP and LDH concentration in supernatants was quantified as before.

Statistics

All data in this study are shown as mean ± SD and were tested for normal distribution. Depending on groups analyzed, statistical analysis was performed by using Student's *t*-test or one-way analysis of variance followed by the Tukey range test for significant differences between the means. Significance was considered for $P < 0.05$. For statistical calculations, GraphPad Prism 5 for Mac OS X, Version 5.0b (GraphPad Software) was used.

Results

Increased cell yield by incorporation of ESWT in the isolation process

To evaluate effects on the isolation efficacy, cells were counted after 19 days in culture, and cell number was normalized to 100 mg nerve wet weight. Sciatic nerve weights ranged between 66.2 and 88.5 mg, and there was no significant difference between the groups (Figure 1B). As shown in Figure 1A, cell yield after 19 days was significantly increased in the ESW treated group. Although initial cell count revealed $1.62 \times 10^6 \pm 9.3\%$ cells per 100 mg nerve wet weight in the control group, cell yield in the ESW-treated group was, on average, 52.3% higher ($3.10 \times 10^6 \pm 8.2\%$ cells per 100 mg nerve wet weight). Furthermore, Figure 2A illustrates a consistent improvement of the cell yield for every culture assessed.

BrdU assay and population doublings per passage reveal a significantly higher proliferation after ESWT for 15 passages

The cell proliferation was quantified using a BrdU assay and assessment of population doublings per passage. In all passages examined (passage 1, 4, 7, 10, 13 and 15), Schwann cells treated with ESWT showed a higher proliferative behavior than the Schwann cells in the control group, respectively (Figure 2). Furthermore proliferation decreased steadily in the control group

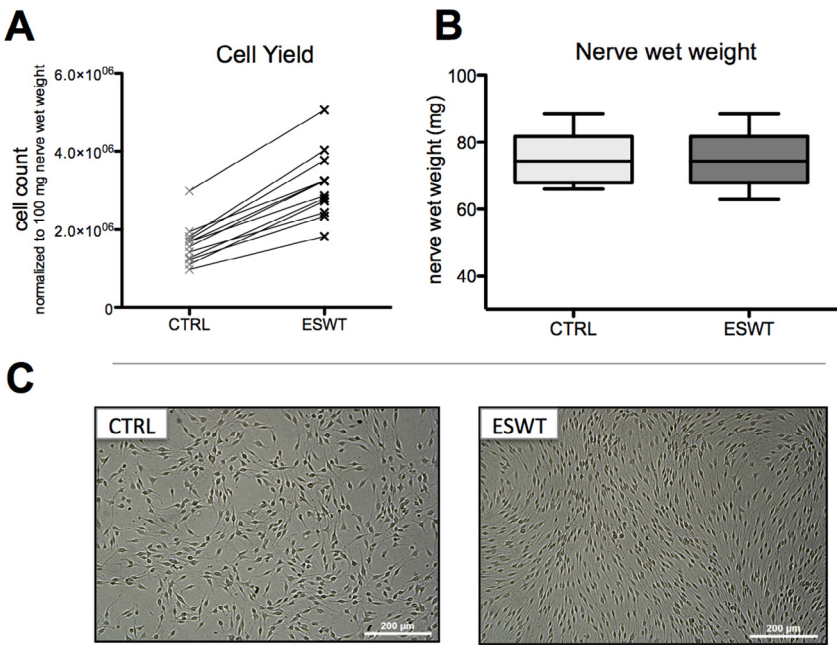

Figure 1. ESWT resulted in an increased cell yield. (A) Intra-animal comparison of Schwann cells counted after 19 days in culture, normalized on 100 mg nerve wet weight, n = 12. (B) nerve wet weight of the respective nerves, assessed with a fine scale before isolation; n = 12. (C) Phase contrast micrographs of SCs in passage 0, untreated control (CTRL) and treated with extracorporeal shockwaves (ESWT).

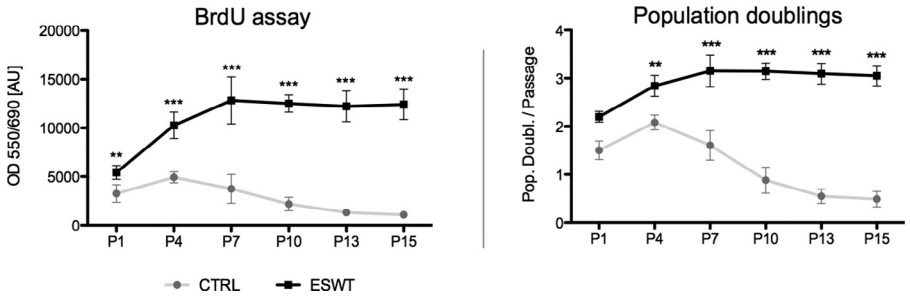

Figure 2. BrdU assay and population doublings per passage revealed a significantly higher proliferation of the ESW treated Schwann cells over 15 passages. BrdU: n = 6 rats, values were obtained in quadruplicates in passage 1, 4, 7, 10, 13 and 15. Population doublings per passage: population doublings were obtained by cell count in passage 1, 4, 7, 10, 13 and 15; n = 6 rats. Data are mean ± SD, and significance was tested with Student's *t* test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. CTRL, untreated control.

starting in passage 4, whereas proliferation in the ESWT group further increased until passage 7 and remained at a similar level until passage 15. Western blots in passage 2, 7 and 15 also exhibited a steady increase in the cell-cycle-arrest/senescence marker $P16^{INK4a}$ in the control group, while ESWT group remained at the same low level for the tested passages (Figure 4).

Sustained purity and P75 expression after ESWT for extended culture period

To determine purity and phenotype of these cultivated cells, expression of Schwann cell specific marker S100b, as well as the markers P75 (proliferative/regenerative phenotype) and P0 (myelinating phenotype), were assessed *in vitro* over 15 passages with flow cytometry (Figure 3). In passage 1, 24.2% more cells expressed S100b in the ESW-treated group compared with the control group (62.6% compared with 38.4%). In both groups, Schwann cell purity increased in passage 2 to 70.0% (ESWT) and 57.3% (CTRL). However, starting in passage 3, S100b expression in the control group decreased steadily over the following passages. In contrast, in the ESW treated group expression increased until passage 5 (85.3%) and remained at this level until passage 15 (Figure 3, S100b). A similar temporal pattern was applied to the expression of the proliferation-associated marker P75: while control group reached a peak expression of 36.7% in passage 3, steadily decreasing over time, ESW-treated cells increased from 57.4% P75 expression to 83.1% positive cells and remained at this level until passage 15 (Figure 3, P75). Myelin marker P0 was down-regulated in both groups; however, it was stronger in the ESWT treated group, exhibiting a significant difference in passage 3 where control group showed an increase of 9.0% compared to passage 2 (Figure 3, P0). Protein expression levels using Western blot densitometric analysis revealed a similar difference between the groups in the expression of S100b as found with flow cytometry in the respective passages

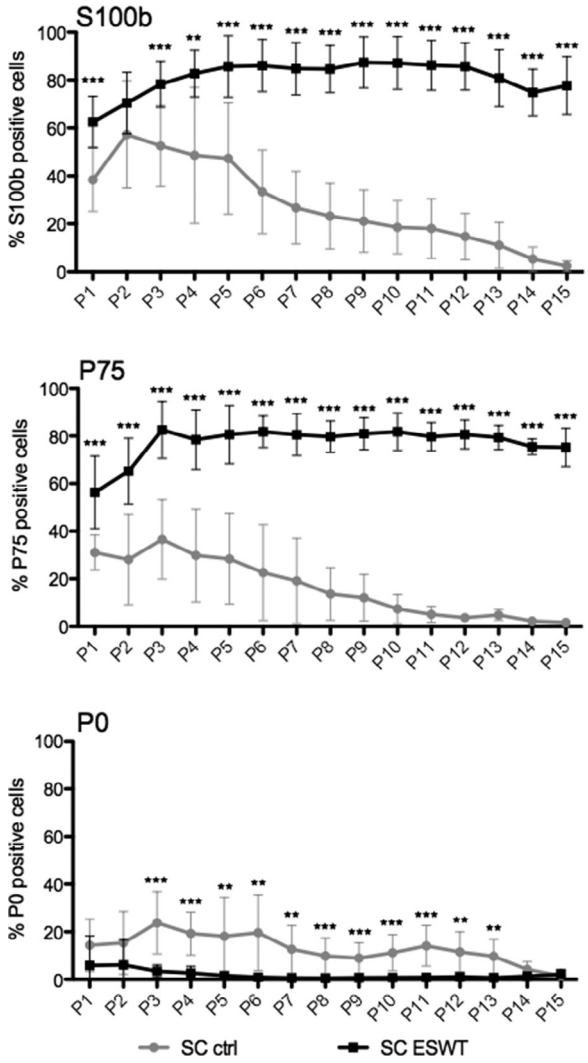

Figure 3. Schwann cells treated with ESW show an increased purity (100b) over 15 passages, along with increased expression of proliferation associated marker P75 and a decreased expression of myelin marker P0. Flow cytometric immunophenotype analysis (S100b, P75, P0) of Schwann cells treated with ESWT (black), compared with control (CTRL; gray) over 15 passages (P1–P15); one passage represents the detachment, counting, and seeding of the cells with 4×10^4 cells/cm^2 for 5 days; n = 10 rats. Data are mean of percentage marker-positive cells ± SD, and significance was tested with Student's *t* test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

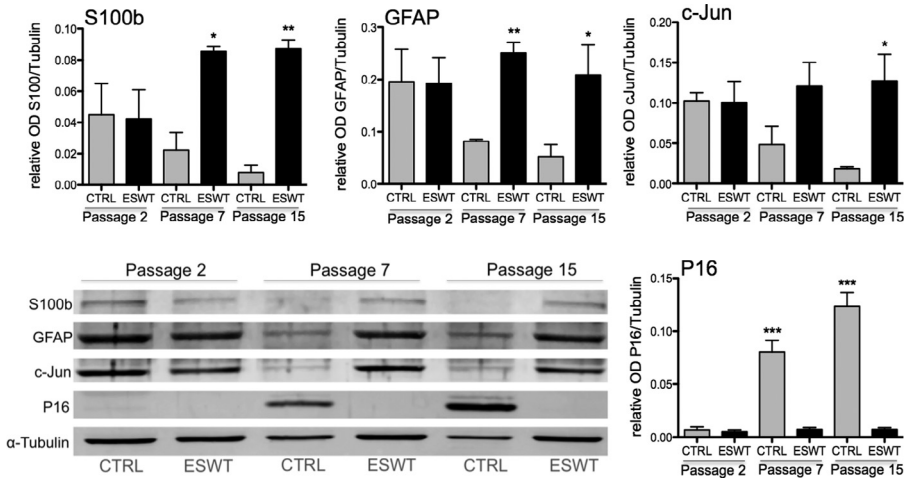

Figure 4. Western blot analysis of Schwann cell protein lysate demonstrates a decrease of Schwann cell markers in the control group along with a simultaneous increase of senescence marker P16. Western blot analysis of Schwann cells in passage 2, passage 7 and passage 15, treated with ESW compared with untreated control (CTRL). Blots were densitometrically analyzed, and data are presented as mean ± SD (n = 6). Statistical significance was tested with one-way analysis of variance and Tukey range test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

passage 2, 7 and 15 (Figure 4, S100). Further analysis of the markers c-Jun and GFAP, both associated with the proliferative Schwann cell phenotype, exhibited an increase of both markers in the ESWT group and a decrease in the control group (Figure 4).

Schwann cells treated with ESWT display a consistent morphology

Schwann cell morphology was assessed in passage 0, 5, 9 and 15 using phase contrast microscopy (Figure 5). Schwann cells of both groups showed a spindle or tripolar shape in passage 0. Starting in passage 5, a second cell morphology was found in the control group, similar to the classic fibroblastic phenotype. Cells exhibiting this morphology increased in number from passage 5 to passage 15 in the control group (Figure 5, indicated with arrows), whereas the ESWT-treated group revealed a homogenous Schwann cell morphology over 15 passages.

Schwann cells revert faster to myelinating phenotype after ESWT

To demonstrate that the proliferative activation of SCs is not permanent and reversible the Schwann cell ability

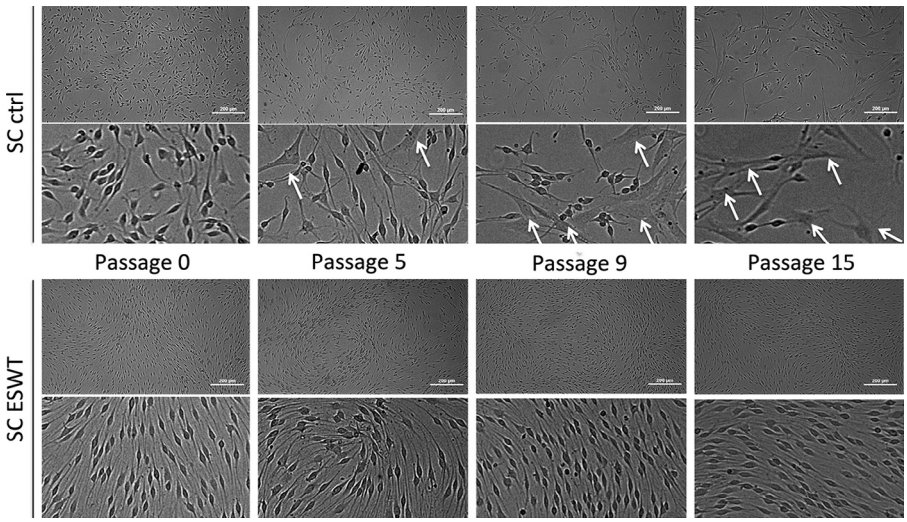

Figure 5. Morphology of Schwann cells changes over time in the control group, whereas it remained consistent in the group treated with extracorporeal shockwaves (ESWT). Phase contrast micrographs depicting Schwann cells in passage 0, 5, 9 and 15 of control group (CTRL, upper bar) and ESW-treated group (lower bar); arrows mark cells displaying non-typical morphology (tripolar, fibroblast-like); size bar indicates 200 µm.

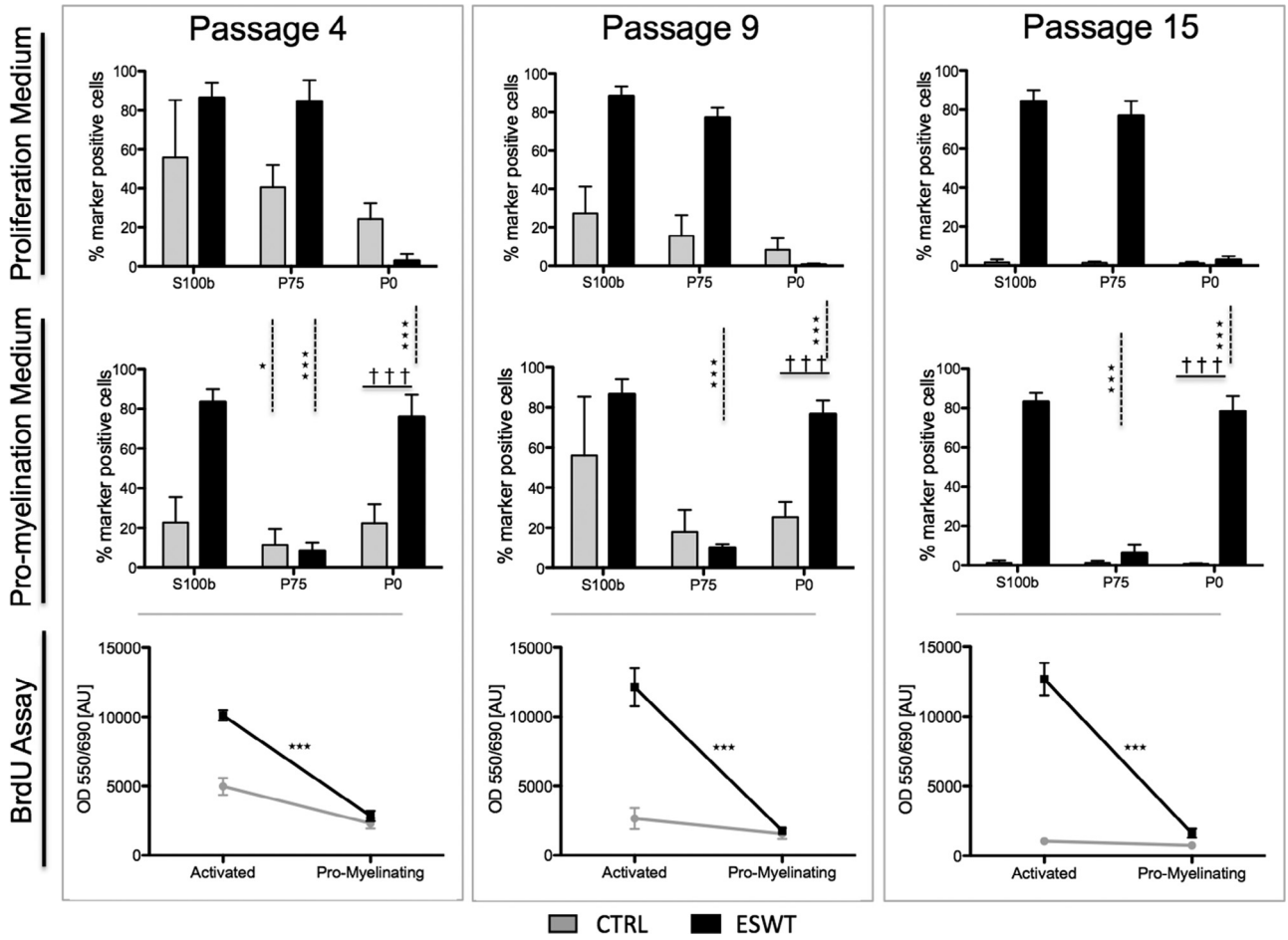

Figure 6. Schwann cells treated with ESWT can express both phenotypes (regenerative and pro-myelinating) dependent on culture medium, but independent of passage number. Flow cytometry analysis concerning expression of Schwann cell markers S100b, P75 and P0 of Schwann cells in activation medium and pro-myelinating medium (day 5 after medium switch). Control group (CTRL) is shown in gray, ESWT group in black. Statistical significance was tested with one-way analysis of variance and Tukey range test. Data are mean ± SD. Comparison of activation to pro-myelination medium of the respective groups: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Comparison between CTRL and ESWT group: $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$. BrdU ELISA of Schwann cells in activation medium and pro-myelination medium; statistical significance was tested with 1-way analysis of variance and Tukey range test. Data are mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; n = 6.

to switch phenotype from proliferating to pro-myelinating was tested. Therefore, in passages 4, 9 and 15, the medium was changed to one lacking pro-proliferative growth factors, giving the cells a minimal stimulus to change their phenotype. Flow cytometry revealed no change in expression of S100b between the cells cultured in the different mediums in the respective passages (Figure 6). In passage 4, cells of the control group and the ESWT group were able to perform the switch, as can be seen in Figure 6: P75 was significantly down-regulated, whereas P0 was strongly up-regulated. Expression of P0, however, was significantly higher in the ESW-treated group. In passages 9 and 15 only, cells in the ESWT group reacted to the stimulus by significant down-regulation of P75 together with up-regulating P0. BrdU assay confirmed a significantly decreased proliferation in the ESW-treated group cultured in pro-myelination medium compared with proliferation medium (Figure 6, lower panel).

ESWT induces immediate and sustained ATP release

Purinergic signaling is capable of inducing enhanced proliferation after ESWT treatment, associated with ATP release [18]. Therefore, we assessed ATP release of the *ex vivo*–treated sciatic nerves. Extracellular concentration of ATP was enhanced depending on the applied energy flux densities. The "minimal" setting with the lowest energy level (0.03 mJ/mm^2) did not result in any initial increase, whereas application of ESW with treatment (0.1 mJ/mm^2) as well as the "maximal" setting (0.19 mJ/mm^2) had a significant increase in initial extracellular ATP as a consequence

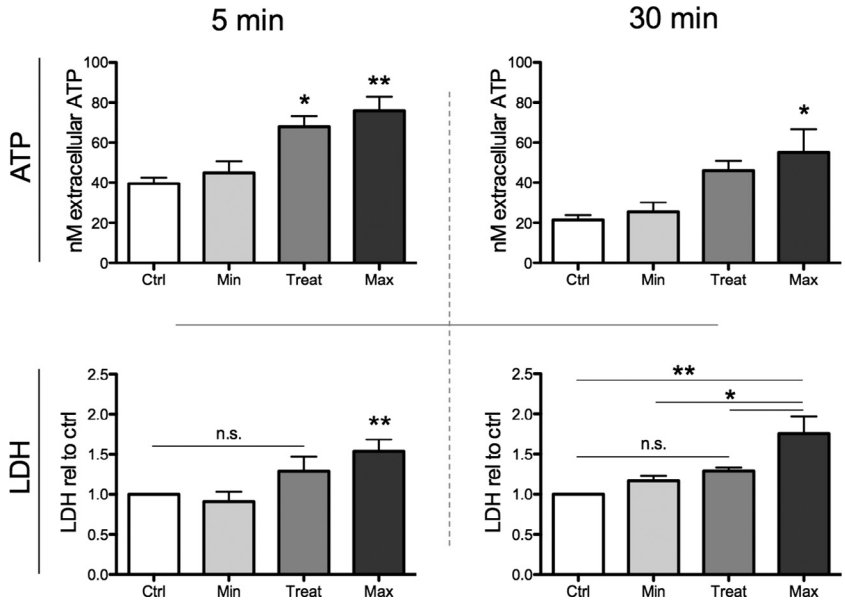

Figure 7. Extracellular ATP was significantly increased in nerves treated with 0.1 mJ/mm^2 (Treat) over a period of 30 min, with no increase in cell damage (LDH); whole nerves without epineurium were treated with ESW of different energy levels: 0.03 (Min), 0.10 (Treat) and 0.19 mJ/mm^2 (Max) and compared with the untreated control (CTRL). ATP concentration in the supernatant was determined after 5 min and 30 min on ice with luminescence, together with assessment of LDH. Amount of ATP was calculated using ATP standards; LDH was normalized to untreated control; n = 5. Statistical significance was tested with one-way analysis of variance and Tukey range test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

(Figure 7, ATP 5 min). Nerve tissue was incubated in freshly added DMEM for 30 min on ice to assess sustained ATP release. These supernatants showed a generally lower concentration of ATP in all groups; however, treatment (0.1 mJ/mm^2) and maximal setting (0.19 mJ/mm^2) again showed higher levels than control and minimal setting (0.03 mJ/mm^2; Figure 7, ATP 30 min). Among other triggers, ATP can be released into the extracellular space due to cell membrane damage. To exclude membrane damage as the reason for increased extracellular ATP after ESW treatment we assessed extracellular LDH concentration as an indicator for cell damage. As seen in Figure 7 (LDH), supernatants of nerve tissue that received the highest energy (0.19 mJ/mm^2) were the only ones that showed significantly enhanced LDH concentrations. At both time points, our selected treatment setting (0.1 mJ/mm^2) did not increase levels of extracellular LDH when compared with the control group.

Discussion

After peripheral nerve injury, Schwann cells are triggered to change their phenotype from myelinating to activated and proliferating and building bands of Büngner, the substrate for outgrowing axons. There are myriad studies describing the importance of Schwann cells during peripheral nerve regeneration [20–22], but the difficulties and issues associated are also well known [23–25]. Particularly in long-distance injuries, the demand for supportive Schwann cells expanded *in vitro* (e.g., seeded on a tubular graft) would be high because autologous Schwann cells lack the proliferative capacity to provide bands of Büngner throughout a tube longer than 40 mm [5]. This limited proliferative capacity is also reflected *in vitro*, displaying together with insufficient purity the main issue of Schwann cell cultures. To our knowledge, we are the first to demonstrate an increase in proliferation, proliferative capacity and purity in *in vitro* Schwann cell cultures with ESWT.

The basis for our study was found in two preceding shockwave studies. Hausner et al. [15] showed that shockwave treatment after nerve dissection leads to significantly accelerated regeneration in a rat model. It was hypothesized that this effect may result from improved macrophage infiltration and an earlier onset of regeneration. However, Weihs et al. [18] elucidate in their study how ESWT stimulates cell proliferation in several cell types (e.g., mesenchymal stem cells) by ATP release-coupled extracellular signal-regulated kinase (ERK) activation. Applying ESWT on the whole nerve before isolation of Schwann cells, our results demonstrate that ESWT is capable of enhancing the extracellular levels of ATP without causing any cell membrane damage. Furthermore, the sustained release over 30 min suggests an active mechanism of ATP release.

Analyzing the cultures in passage 0, we assessed a significantly higher cell yield in all cultures treated

with ESWT (Figure 1), alongside a significantly increased proliferation rate in passage 1 (Figure 2). In the shockwave-treated group, quality of culture, reflected in purity and proliferation rate, improved over the first passages: S100b, a marker indicating purity of Schwann cells, increased over the first five passages and remained at the level for the period of analysis; this purity was also observed in morphology of Schwann cells (Figure 4); moreover, P75 increased over time, whereas P0 in the same manner decreased until passage 4 and subsequently was not expressed (Figure 3); P0 and P75 are known to counteract each other [26]: P0 is a myelin component and therefore solely expressed in myelinating Schwann cells [27], whereas P75 is a marker associated with the regenerative, proliferating phenotype of Schwann cells [28,29]. Western blot analysis at three time points throughout the culture period revealed a similar expression pattern for S100b, acting as a control marker between the methods (Figure 4, compared with Figure 5 S100b). GFAP and c-Jun were also up-regulated in passage 7 (compared with passage 2) and showed a comparable expression in passage 15. Both markers are associated with the regenerative phenotype. GFAP is suppressed in myelinated axons and becomes upregulated after injury, initiating proliferation by binding of integrin αvβ8 [30]. The transcription factor c-Jun however is an antagonist to Krox20, a protein controlling the myelination in the peripheral nervous system, and displays one of the key regulators to initiate and maintain the regenerative phenotype [31,32]. Together with the strongly increased proliferation (assessed with BrdU assay and population doubling per passage), the Schwann cells treated with ESWT represent a highly regenerative phenotype for an extended culture period of 15 passages (103 days).

As the sum of these observations leads inevitably to the question whether hyper-proliferating Schwann cells may lead to adverse effects such as post-stimulus proliferation and subsequently Schwannoma formation, we conducted a functionality experiment. Schwann cells were cultured for 5 days in basic medium lacking any kind of proliferation stimulating growth factors (forskolin, pituitary extract). Schwann cells treated with ESWT reacted even more to the change of stimulus, independent of the passage: they not only stopped proliferating (Figure 6, BrdU) but also significantly down-regulated the proliferation-associated marker P75 and up-regulated the myelin component P0. This prompt and consistent reaction to the absence of mitogenic growth factors demonstrates their capacity to switch to the myelinating phenotype. However, the functionality of the SCs in an *in vivo* defect model has to be shown.

In contrast to the ESWT group, the untreated Schwann cells did not display a consistent Schwann cell phenotype over the culture period of 15 passages. Starting with passage 5 to 7, purity and proliferation significantly decreased. Abated purity was observed in lower expression of S100b (Figures 3 and 6), as well as in increased appearance of cells displaying an atypical morphology (Figure 5). Reduced proliferation was primarily assessed in a decline of population doublings and additionally by reduced BrdU OD values. The entire marker expression levels associated with the regenerative phenotype (P75, GFAP, c-Jun) diminished over time, whereas the senescence marker P16 significantly increased. Therefore, our *in vitro* study reflects the before mentioned *in vivo* problem and represents a possible solution: the limited proliferative capacity of Schwann cells can be improved with ESWT. With treated Schwann cells building a growth substrate faster and for a longer period of time, the *in vivo* effect can be twofold: ESWT not only results in an acceleration of regeneration by activating autologous Schwann cells, as has been shown by Hausner et al. [15], but would also allow reimplantation of a high number of autologous Schwann cells expanded *ex vivo* in a decidedly regenerative state.

A partial explanation for the underlying mechanisms of the observed effects could be the sustained ATP release. A wide range of mechanisms from vesicular release over connexins/pannexins to ABC transporters are thought to conduct an active release of ATP [33–35]. Subsequent purinergic signaling plays a crucial role not only as a danger-associated molecular pattern but also in a variety of cellular functions such as proliferation, chemotaxis, differentiation and amplification of other signals [18,36]. This includes Schwann cell–axon interactions. Immature/unmyelinating Schwann cells in particular signal via extracellular ATP in a paracrine manner with axons [37,38]. It was proposed that ATP and glutamate are building a positive feedback loop enhancing their activities [39]. The fate of Schwann cells is influenced by neuronal activity, by the activation of purinergic metabotropic P2Y receptors and direct actions of ATP and its metabolite adenosine [40–42], as well as by the activation of metabotropic glutamate receptors [43]. Furthermore, purinergic signaling is thought to be both an autocrine and a paracrine amplifier for other signaling inputs. This is a further explanation for the enhanced proliferative activity of ESWT-treated Schwann cells in medium-containing proliferation-inducing factors such as forskolin and pituitary extract. Adenosine, a metabolic product of ATP hydrolization, is hypothesized to play a role in learning by affecting histone-modifying proteins resulting in epigenetic changes [44]. Therefore, the prolonged phenotypic stability and increased susceptibility to external stimuli as observed in the phenotype

switch experiments in this study may be explained by epigenetic changes.

In conclusion, we observed a higher proliferative activity without phenotype commitment, increased purity of culture and reduced expression of senescence-associated markers even after long cultivation periods. These positive effects of ESWT on Schwann cell isolation and cultivation may partly be explained by the actions of extracellular ATP. However, to gain a deeper understanding of the effects of ESWT on Schwann cells and their natural habitat, the nerve, additional *in vitro* and *in vivo* studies focusing on purinergic signaling, mechanotransduction and epigenetic processes have to be performed.

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