Cytotherapy, 2016; 18: 760–770

International Society for Cellular Therapy
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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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(Received 18 January 2016; accepted 5 March 2016)

ISSN 1465-3249
http://dx.doi.org/10.1016/j.jcyt.2016.03.002

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

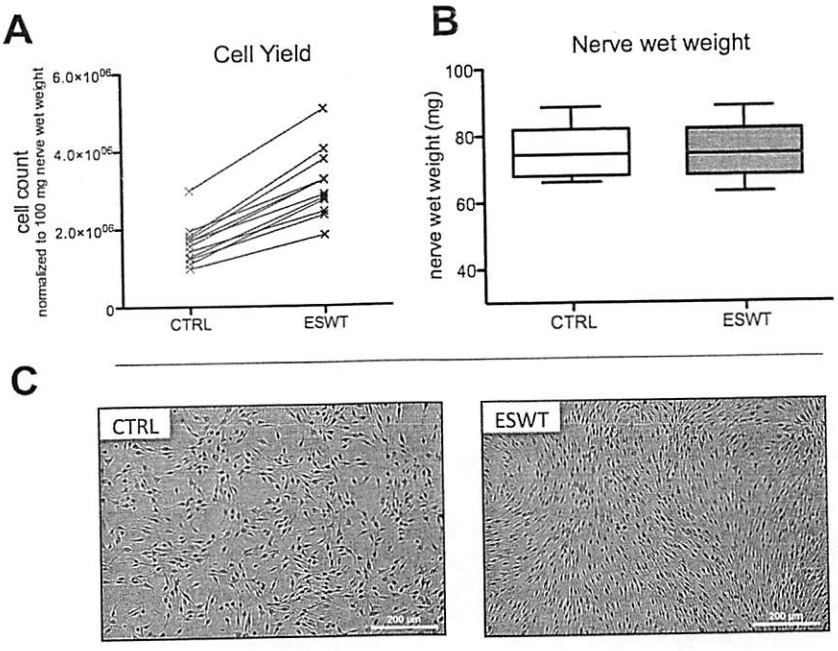

Figure 1. ESWT resulted in an increased cell yield. (A) Intra-animal comparison of Schwann cells counted after 19 days in culture, normalized on 100 mg nerve wet weight, n = 12. (B) nerve wet weight of the respective nerves, assessed with a fine scale before isolation; n = 12. (C) Phase contrast micrographs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