

Shock Wave Treatment Protects From Neuronal Degeneration via a Toll-Like Receptor 3 Dependent Mechanism: Implications of a First-Ever Causal Treatment for Ischemic Spinal Cord Injury

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Background—Paraplegia following spinal cord ischemia represents a devastating complication of both aortic surgery and endovascular aortic repair. Shock wave treatment was shown to induce angiogenesis and regeneration in ischemic tissue by modulation of early inflammatory response via Toll-like receptor (TLR) 3 signaling. In preclinical and clinical studies, shock wave treatment had a favorable effect on ischemic myocardium. We hypothesized that shock wave treatment also may have a beneficial effect on spinal cord ischemia.

Methods and Results—A spinal cord ischemia model in mice and spinal slice cultures *ex vivo* were performed. Treatment groups received immediate shock wave therapy, which resulted in decreased neuronal degeneration and improved motor function. In spinal slice cultures, the activation of TLR3 could be observed. Shock wave effects were abolished in spinal slice cultures from TLR3^{-/-} mice, whereas the effect was still present in TLR4^{-/-} mice. TLR4 protein was found to be downregulated parallel to TLR3 signaling. Shock wave-treated animals showed significantly better functional outcome and survival. The protective effect on neurons could be reproduced in human spinal slices.

Conclusions—Shock wave treatment protects from neuronal degeneration via TLR3 signaling and subsequent TLR4 downregulation. Consequently, it represents a promising treatment option for the devastating complication of spinal cord ischemia after aortic repair. (*J Am Heart Assoc.* 2015;4:e002440 doi: 10.1161/JAHA.115.002440)

Key Words: neuronal degeneration • shock wave therapy • spinal cord ischemia • Toll-like receptors

Paraplegia or paraparesis due to spinal cord ischemia still represents a devastating complication of both aortic surgery and endovascular aortic repair, without any causal treatment option.^{1,2} The incidence of postoperative paraplegia can approach up to 15% in high-risk patients.³ Protection of

the spinal cord is commonly achieved by moderate or deep hypothermia under circulatory arrest⁴ and pharmacological neuroprotection.^{5,6} Monitoring of spinal cord function is achieved by motor evoked potentials⁷ or infrared spectroscopy.⁸ A further preventive and curative approach includes cerebrospinal fluid drainage.⁹ The rationale is to decrease cerebrospinal pressure that is highly elevated because of tissue swelling as an ischemic inflammatory response.

Immediate paraplegia occurs due to neuronal apoptosis or necrosis of motor neurons and is rarely reversible. Besides acute paraplegia directly after surgery, delayed-onset paraplegia has been observed.¹⁰ Toll-like receptor (TLR) 4-dependent microglial activation has been identified in this setting to mediate spinal cord ischemia–reperfusion injury.¹¹ Microglia are spinal resident macrophages and the main cell type in the resting central nervous system that expresses TLR3 and TLR4.^{12,13} TLR4 activation has even been shown to trigger neurodegeneration and is thought to be involved in the pathogenesis of several kinds of neurological disorders such as Alzheimer's disease.¹⁴ The absence of TLR4 in TLR4-knockout mice showed neuroprotective effects against focal

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cerebral ischemia. This effect was missing in TLR3-knockout animals.¹⁵ Moreover, the TLR3 agonist polyinosinic:polycytidylic acid [poly(I:C)] has been described as having a protective effect against cerebral ischemia–reperfusion injury through the downregulation of TLR4 signaling via TLR3 activation.¹⁶ In previous work, we showed that shock wave therapy (SWT), a relatively new treatment approach, mediates regeneration of ischemic muscle via TLR3 signaling and simultaneously causes TLR4 downregulation.¹⁷ TLR3 is the only TLR without signaling via myeloid differentiation primary response gene (MyD88). It is mediated via the adaptor molecule TIR-domain-containing adapter-inducing interferon- β (TRIF), and the transcription factor IRF3, which finally initiates transcription of interferons and interferon-induced genes. TLR4 signaling can be mediated by the adaptor molecule MyD88, TNF receptor associated factor 6 (TRAF6) and subsequent phosphorylation of the transcription factor p65, which initiates nuclear factor- κ B transcription; however, it can also be mediated via TRIF, like TLR3. Consequently, the 2 receptors have a common signaling pathway and are able to interact. Only recently it was shown that TLR3 activation via its agonist poly(I:C) results in decreased levels of TLR4, MyD88, and nuclear factor- κ B; however, poly(I:C) did not downregulate TLR4/MyD88 in TLR3^{-/-} mice. In addition, poly(I:C) treatment decreased tumor necrosis factor α and interleukin (IL) 1b serum levels in mice stimulated with the TLR4 agonist lipopolysaccharide (LPS).¹⁸ These results clearly state an interaction between these 2 receptors; however, the exact working mechanism of interaction is not fully understood.

We hypothesized that shock waves have a regenerative or at least protective effect in acute spinal cord ischemia.

Shock waves are specific sound pressure waves with the capability to induce tissue-regenerative effects.¹⁹ This new treatment option was developed as an alternative to or as standard of care for nonhealing bone fractures, wound healing disturbances, and tendinopathies.^{20–22} Moreover, our group developed low-energy SWT for open-heart direct epicardial application after myocardial infarction.²³

In this way, TLR3 stimulation and downstream signaling leads to the production of cytokines and chemokines that in turn modulate a macrophage-mediated inflammatory response. This effect seems to be prerequisite for the induction of angiogenesis via the release of pivotal angiogenic growth factors vascular endothelial growth factor (VEGF) and placental growth factor. Life Technologies, USA.^{23–25} New arterioles are formed as a long-term effect, leading to regeneration of the ischemically harmed tissue.

Tissue regeneration represents a major field of medical research, especially in cardiac repair. Current approaches include (stem) cell- or gene-based therapies^{26–29}; however, neither has gained broad clinical use due to the complexity of

application and distinct side effects. A new method that is safe and feasible and that has been used in medicine for years, such as shock wave treatment, could develop a straightforward approach to clinical tissue regeneration.²³

With a focus mainly on the myocardium, spinal cord ischemia was nearly neglected in regenerative research as an “orphan disease.” Given our promising results from cardiac applications, we aimed to investigate whether shock wave treatment would have a protective or perhaps a regenerative effect on spinal cord ischemia.

Methods

Animals

Experiments were approved by the Austrian animal care and use committee. The investigation conformed to the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Investigators blinded to the treatment of the animals performed measurements and analyses.

Male C57BL/6 wild-type mice (Charles River, Sulzfeld, Germany) aged 12 to 14 weeks and weighing 25 to 30 g were randomly divided into 6 groups: control (CTR) 24 hours, CTR 72 hours, CTR 7 days, SWT 24 hours, SWT 72 hours, and SWT 7 days. The sample size of each group at each time point was 6. Shock wave treatment was applied immediately after surgery, whereas control animals were left untreated. During the experiments, mice were housed under standard conditions with a 12-hour light/dark cycle. Water and commercial mouse diet were available ad libitum.

Spinal Cord Ischemia Model and Laser Doppler Measurement

Anesthesia was administered by an intraperitoneal injection of ketamine (80 mg/kg body weight; Graeb) and xylazine hydrochloride (Rompun; 5 mg/kg body weight; Bayer). Mice were endotracheally intubated and mechanically ventilated. Aortic cross-clamping was performed, as described previously.^{30,31} Briefly, a left lateral thoracotomy in the second intercostal space was performed. After the exposition of the aortic arch and the subclavian artery, the aortic arch was cross-clamped between the left carotid artery and the left subclavian artery using a murine vascular clamp. An additional clamp was placed at the origin of the left subclavian artery to avoid retrograde perfusion via the vertebral artery. For the same reason, the left mammary artery was destroyed by electrical cauterization. The clamps were removed after 10 minutes.

Blood flow measurements were performed prior to surgery and during ischemia using a laser Doppler perfusion image analyzer (Moor Instruments). To minimize data variables

attributable to temperature, mice were kept on a heating plate at 37°C before measurement.

Animals were sacrificed at 24 and 72 hours, respectively, 7 days after surgery. Spinal cord samples were obtained, as described previously.³¹ Subsequently, they were rinsed in sterile saline and divided into equal halves, 1 part of which was placed in 4% paraformaldehyde overnight for subsequent paraffin embedding; the other part was snap frozen in liquid nitrogen followed by RNA extraction for reverse transcription–polymerase chain reaction analysis.

Shock Wave Treatment

Immediately after spinal cord ischemia surgery, a single shock wave treatment was performed in the treatment group while animals were still under anesthesia, whereas control animals received sham treatment. The commercially available Orthogold device with applicator CG050-P (Tissue Regeneration Technologies LLC) was used for SWT. Overall, 500 impulses at an energy flux density of 0.1 mJ/mm² and a frequency of 5 Hz were applied to the entire spinal column. Common ultrasound gel was used for coupling of the shock wave applicator.

Neurological motor tests

Neurological motor function was assessed 24 hours, 72 hours, and 7 days after surgery by a single researcher blinded to the treatment. Standardized tests to evaluate mobility, coordination, strength, and speed were used, as described previously.³²

Grip strength test

For the evaluation of limb strength, mice were placed on a cage lid. Subsequently, the lid was inverted and elevated at standard height of 10 cm. The time that the mouse remained clinging to the inverted cage lid was measured. The test was repeated 3 times with each mouse. The best result was kept for statistical analysis. The highest value of the 3 measurements was defined as the best of 3 results.

Cylinder test

To assess motor forelimb impairment after spinal cord ischemia, mice were placed in an acrylic cylinder and observed for 3 minutes. The number of upright wall contacts during this time was counted. Results are shown as the number of upright wall contacts.

Beam-walking test

For the evaluation of motor coordination and balance deficits, mice were placed at the beginning of a walking

beam that was 80 cm long and 3 cm wide. The task of the mice was to walk along the beam toward the end. Three trials were performed per mouse. The time necessary to reach the end of the beam and the number of lateral foot slips per mouse were assessed. The best of the 3 results was kept for statistical analysis. The least time and the fewest slips of the 3 measurements were defined as the best of 3 results.

Locomotor activity test

To evaluate the motor activity and movements of a mouse in an open area, a rat cage was divided into 15 equal squares. The mouse was placed in the middle of the cage, and the number of squares that the mouse passed with all 4 limbs within 30 seconds was counted.

Spinal Slice Culture

Spinal slice culture was performed, as described previously.³³ Briefly, spinal cords from 12-week-old C57BL/6 wild-type mice (Charles River, Sulzfeld, Germany) and from TLR3^{-/-} mice (provided by Prof. Shizuo Akira, Osaka University, Osaka, Japan; bred at the animal facility at Innsbruck Medical University) and TLR4^{-/-} mice (obtained from Charles River, Sulzfeld, Germany; bred at the animal facility at Innsbruck Medical University) were harvested and dissected into 1-cm slices (4 slices per group from 2 different animals). Spinal cord slices (including human samples) were cultured overnight in DMEM high glucose (Gibco; Life Technologies) containing 10% (vol/vol) fetal calf serum and 1% penicillin-streptomycin solution (both from Sigma-Aldrich) and 5 ng/mL mouse nerve growth factor (Alomare Laboratories). After 24 hours, culture medium was changed to DMEM low glucose (1 g glucose, Gibco; Life Technologies) containing penicillin, streptomycin, and glutamate (Sigma-Aldrich), and spinal slices were subjected to hypoxia for 24 hours in a common hypoxia chamber. Following this, culture medium was changed again to the DMEM high glucose medium, and the SWT group received shock wave treatment (300 impulses with an energy flux density of 0.08 mJ/mm² at a frequency of 3 Hz). Groups were harvested after 24 and 48 hours. Spinal slice cultures were repeated in duplicate.

Spinal slices were snap frozen in liquid nitrogen. After homogenization, protein-balanced samples were separated in 10% SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes. After blocking with 5% BSA in TBS 0.1% Tween, blots were incubated with polyclonal TLR3 antibody (rabbit-TLR3 antibody, 80 kDa, Sigma SAB2900405; Sigma-Aldrich) and TLR4 antibody (rabbit anti-TLR4 antibody, 90 kDa, ab 13867; Abcam) at a dilution of 1:1000 overnight at 4°C. For secondary antibody, a goat antirabbit antibody

was used at a dilution of 1:1000 in 5% BSA 1× TBS 0.1% Tween and incubated for 1 hour at room temperature. For visualization, 1 mL enhanced chemiluminescence (Pierce ECL; Thermo Fisher Scientific) was used per membrane.

Human samples

This study used a nonembalmed cadaver with a postmortem time of 2 hours, which was defined as spinal cord ischemia time. The human cadaver (male) was donated to the Division of Clinical and Functional Anatomy, Department for Anatomy, Histology, and Embryology of the Medical University of Innsbruck. Informed consent was given for use of the cadaver for scientific and educational purposes.³³ Sampling was performed from the upper cervical segments of the spinal cord.

Histological Analysis and Immunofluorescence Staining

Initially, 5- μ m tissue paraffin sections were deparaffinized. Subsequently, heat-mediated antigen retrieval using unmasking solution (Vector antigen unmasking solution; Vector Laboratories) was performed according to the manufacturer's protocol. Samples were blocked with a mixture of 1% BSA for 30 minutes at room temperature. For specific CD31 (rabbit antimouse; Abcam) staining, slides were incubated with the primary antibody overnight at 4°C. Likewise, Iba1 staining (rabbit antimouse; Wako) was performed. For signal detection, the secondary antibody (goat antirabbit IgG, Alexa Fluor 568; Life Technologies) was incubated for 1 hour at room temperature. Nuclear staining was performed using DAPI (Life Technologies, USA), according to the manufacturer's protocol. The slides were mounted in Mowiol (Mowiol 4-88; Carl Roth). In addition, staining with Fluoro-Jade B (Millipore) was performed, as recommended by the manufacturer. Analysis of 5 slides per animal was performed in a blinded fashion. For each slide, 5 randomly chosen fields at $\times 200$ magnification were photographed. For quantification, images were taken with an Olympus IX70 microscope. Quantification was done by counting the number of capillaries, degenerating neurons, and macrophages per high-power field using ImageJ (National Institutes of Health). All slides were analyzed by a single blinded researcher.

Real-Time Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from homogenized tissue using the RNeasy Kit (Machery-Nagel), according to the manufacturer's instructions. Real-time reverse transcription–polymerase chain reaction for gene expression analysis was performed

with the ABI PRISM 7500 Sequence Detection System (Applied Biosystems; Life Technologies). The following primers were designed using Primer Express software (Applied Biosystems; Life Technologies): IL-6 5' TCC AGA AAC CGC TAT GAA GTT CC 3' (S); 5' GTC ACC AGC ATC AGT CCC AAG 3' (AS); 5' CTC TGC AAG AGA CTT CCA TCC AGT TGC CT3' (probe); IL-10 (S) 5' GAA GAC CCT CAG GAT GCG G 3', (AS) 5' CCT GCT CCA CTG CCT TGC T 3', probe 5'CGC TGT CAT CGA TTT CTC CCC TGT GA 3'; transforming growth factor β (S) 5' CCC TGC CCC TAT ATT TGG A 3', (AS) 5' ACG GTG ACG CCG AAG CGC 3', probe 5' CAC ACA GTA CAG CAA GGT CCT TGC CCT 3'. For VEGF and hypoxia inducible factor 1 α , or HIF-1 α , Assays on Demand were used (VEGF: Mm01281449_m1; HIF-1 α : Mm00468869_m1; Life Technologies). The polymerase chain reaction was performed in a final volume of 25 μ L containing 1 μ L cDNA, 12.5 μ L Master Mix (Applied Biosystems; Life Technologies), 1 μ L fluorogenic hybridization probe, 6 μ L primer mix, and 5.5 μ L distilled water. The amplification consisted of a 2-step polymerase chain reaction (40 cycles; 15-second denaturation step at 95°C and 1-minute annealing/extension step at 60°C). Specific gene expression was normalized to the housekeeping gene β -actin, given by the formula $2^{-\Delta Ct}$. The result for the relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The mean Ct values were calculated from double determinations, and samples were considered negative if the Ct values exceeded 40 cycles.

Statistical Analysis

All results are expressed as mean \pm SEM. Statistical comparisons between 2 groups were performed by Mann–Whitney test. Repeated-measures ANOVA was used for the analysis of functional outcome. The comparison of functional outcomes needs to be interpreted with care because only mice that survived throughout the experiment could be assessed, leading to an unavoidable selection bias. For the survival analysis, animals were visited daily, and the number of survivors was assessed for 7 postoperative days in the morning. Results are represented as Kaplan–Meier survival curves. A log-rank test was performed for the comparison of survival. Only animals sacrificed on day 7 were included in the survival analysis. Probability values <0.05 were considered statistically significant.

Results

SWT Protects From Neuronal Degeneration

Laser Doppler perfusion imaging was performed during aortic cross-clamping (between left carotid and left subclavian arteries) to confirm that a complete decline of the entire

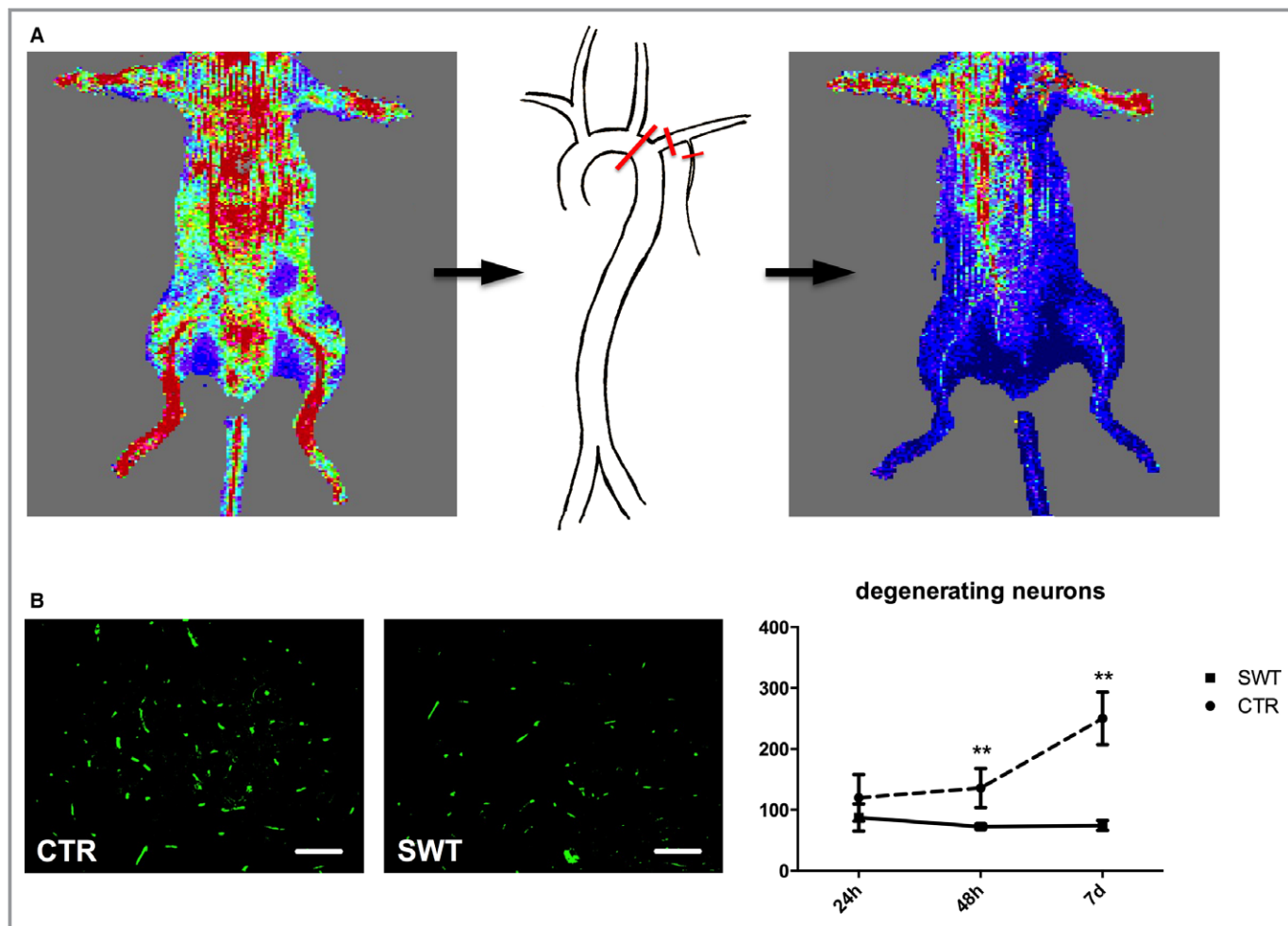


Figure 1. Shock wave therapy protects from neuronal degeneration. A, Aortic cross-clamping was performed between the left carotid and subclavian arteries. Due to collateral blood supply, the left subclavian artery also had to be clamped. To avoid perfusion of the thoracic spine via intercostal arteries due to retrograde flow from the vertebral artery, the left mammary artery was destroyed by cauterization. This finally resulted in complete decline of the entire lower body blood flow. B, Fluoro-Jade B immunostaining revealed higher numbers of degenerating neurons in the untreated controls at 48 hours and 7 days after treatment, showing a progressive trend. In shock wave–treated animals, the number of degenerating neurons remained stable over time. Caliber represents 20 μm , ** $P < 0.01$. CTR indicates control; d, days; h, hours; SWT, shock wave therapy.

lower body blood flow was achieved. Due to collateral blood supply, the left subclavian artery also had to be clamped. To avoid perfusion of the thoracic spine via intercostal arteries due to retrograde flow from the vertebral artery, the left mammary artery was destroyed by cauterization (Figure 1A). SWT was applied immediately after opening of the cross-clamps. Immunofluorescence staining with Fluoro-Jade B was performed to label degenerating neurons. Quantification at different time points revealed that there was no significant difference between treatment and control groups after 24 hours (number of degenerating neurons per high-power field for CTR versus SWT: 119.89 ± 38.29 versus 87.47 ± 22.46 , $P = 0.33$), whereas the number of degenerating neurons was significantly higher 48 hours after ischemia without SWT (CTR versus SWT: 149.52 ± 32.34 versus 75.50 ± 2.87 ,

$P = 0.0025$) and continued increasing up to 7 days after ischemia without SWT (CTR versus SWT: 250.20 ± 42.98 versus 74.50 ± 8.14 , $P = 0.0087$) (Figure 1B). Even more impressive was the fact that the number of degenerating neurons in the treatment group remained stable over time. Consequently, neurons that were not damaged as early as 24 hours after injury did not become injured later in the SWT group.

SWT Modulates Inflammatory Response

Inflammation is an integral part of every ischemic process. Inflammation after spinal cord ischemia contributes to remodeling and thus is crucial for regeneration. SWT was described as modulating inflammation¹⁵; therefore, we measured mRNA expression of IL-6, which is known to be

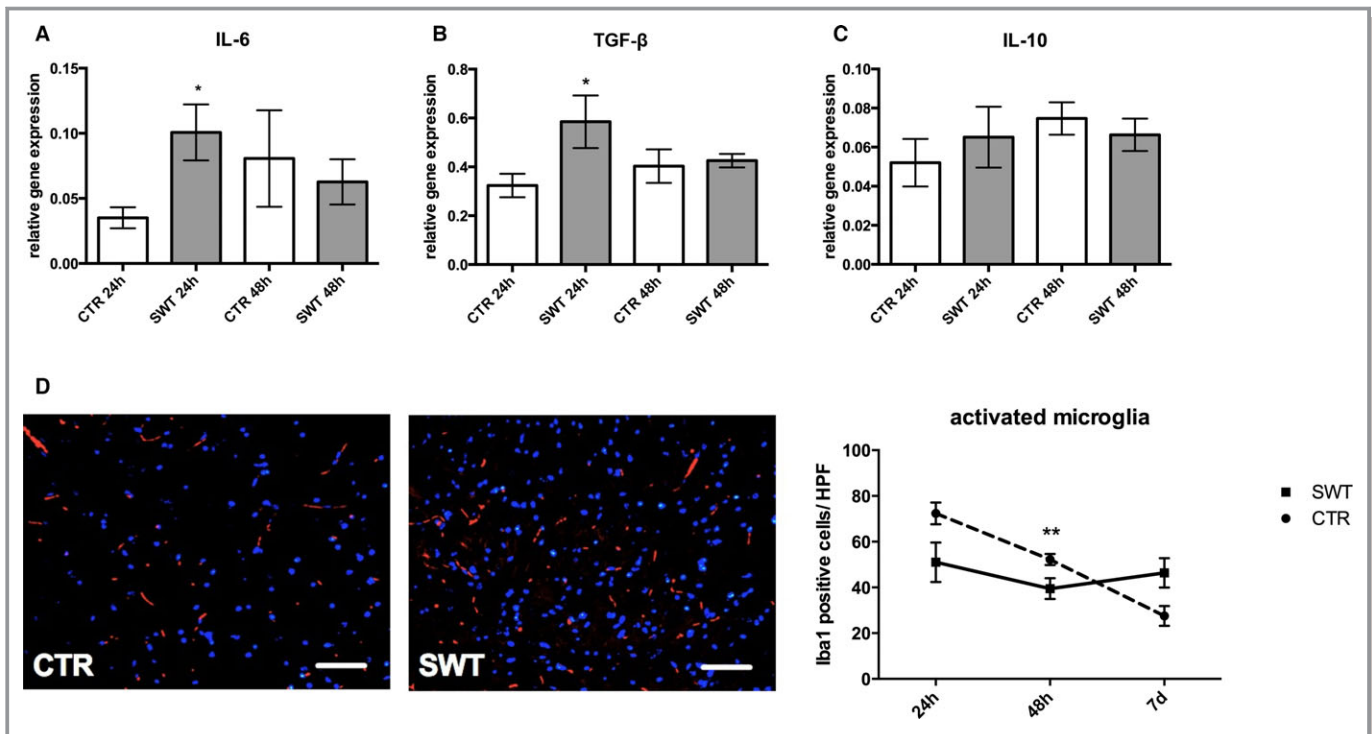


Figure 2. Shock wave therapy modulates inflammatory response. A, An early increase of IL-6 mRNA was found after 24 hours. IL-6 is responsible for monocyte recruitment and represents the initial proinflammatory response toward regeneration of the ischemically harmed tissue. $*P<0.05$. B, Early autocrine signaling of TGF- β mRNA could be observed in the treatment group. TGF- β is secreted by macrophages. This finding is in line with the increase of IL-6. $*P<0.05$. C, No relevant alteration was observed in the anti-inflammatory cytokine IL-10. Maybe its limiting effect toward inflammation could be detected at a later time point. D, Activation of microglia that represent macrophages of the spinal cord was proven by Iba1 immunostaining and revealed a more stable condition of shock wave-treated animals over time. The number of activated microglia was higher in the untreated controls at earlier time points, demonstrating severe tissue injury. Finally, at 7 days after injury, the amount of activated microglia was lower than in the treatment group, indicating less response on loss of tissue. Caliber represents 20 μ m, $**P<0.01$. CTR indicates control; d, days; h, hours; HPF, high-power field; IL, interleukin; SWT, shock wave therapy; TGF- β , transforming growth factor β .

responsible for an early proinflammatory response after SWT and thereby causes recruitment of macrophages. IL-6 was significantly increased after 24 hours in the SWT group, marking an early proinflammatory phase (relative gene expression for CTR versus SWT after 24 hours: 0.04 ± 0.01 versus 0.09 ± 0.02 , $P=0.029$). This effect was observed in non-SWT-treated mice after only 48 hours (CTR versus SWT: 0.08 ± 0.04 versus 0.06 ± 0.02 , $P>0.99$) (Figure 2A). In line with early IL-6 increase, we found transforming growth factor β significantly increased after 24 hours (relative gene expression for CTR versus SWT after 24 hours: 0.32 ± 0.05 versus 0.58 ± 0.1 , $P=0.03$). Transforming growth factor β is secreted by macrophages and activates them in an autocrine manner. Again, the effect of SWT was already gone after 48 hours (CTR versus SWT: 0.4 ± 0.07 versus 0.43 ± 0.03 , $P>0.99$) (Figure 2B). IL-10 is known as an opponent of IL-6 and exerts an anti-inflammatory effect. No alteration in IL-10 mRNA expression could be found, confirming the prior findings of macrophage (ie, microglia) recruitment (relative gene expression for CTR versus SWT after 24 hours:

0.05 ± 0.01 versus 0.07 ± 0.01 , $P=0.56$; after 48 hours: 0.07 ± 0.01 versus 0.07 ± 0.01 , $P=0.41$) (Figure 2C).

Microglia represent spinal cord resident macrophages. Their amount of activation can be seen as an indirect sign of the degree of injury, in contrast to the previously mentioned regenerative effect of inflammation. The number of activated microglia was significantly higher in untreated controls 48 hours after therapy, demonstrating severe tissue damage (Iba1-positive cells per high-power field for CTR versus SWT after 24 hours: 72.36 ± 4.73 versus 46.89 ± 9.24 , $P=0.065$; after 48 hours: 52.23 ± 2.40 versus 39.48 ± 4.55 , $P=0.0043$). Finally, 7 days after injury, the amount of activated microglia was lower than in the treatment group, indicating less response on loss of tissue, although not significant (CTR versus SWT: 27.55 ± 4.35 versus 46.37 ± 6.4 , $P=0.0519$). In contrast, the amount of microglia in the SWT group remained constant over time (Figure 2D).

Lower numbers of microglia at an early time point in the SWT group seem to be contradictory to the IL-6 increase at 24 hours after SWT; however, in contrast to the control group,

there was an increase of IL-10 in the SWT group at the same time point. This may explain why the number of microglia was not altered in the treatment group and thus represents the modulated inflammatory response on shock wave treatment.

SWT Induces Angiogenesis by Capillary Sprouting

Hyperemia is part of every inflammatory process and is necessary to supply the tissue with reparative and immune cells. As an underlying mechanism, acute capillary sprouting occurs; therefore, we measured mRNA expression of the 2 major angiogenic markers VEGF and HIF-1 α . Indeed, VEGF expression (relative gene expression for CTR versus SWT after 24 hours: 0.15 ± 0.05 versus 0.45 ± 0.15 , $P=0.191$; after 48 hours: 0.05 ± 0.005 versus 0.18 ± 0.04 , $P=0.024$) and HIF-1 α expression (relative gene expression for CTR versus SWT after 24 hours: 0.04 ± 0.01 versus 0.16 ± 0.05 , $P=0.057$; after 48 hours: 0.02 ± 0.01 versus 0.14 ± 0.01 , $P=0.036$) were significantly increased in the treatment group compared with untreated controls (Figure 3A and 3B).

To verify whether this angiogenic response truly resulted in higher numbers of vessels, we performed immunostaining for CD31-positive capillaries. In fact, there was an impressive early increase of capillaries 24 hours after treatment (capillaries per high-power field for CTR versus SWT: 2.35 ± 0.75 versus 11.45 ± 1.38 , $P<0.0001$). A lower but nevertheless significant part of this effect was still present after 7 days (CTR versus SWT: 1.474 ± 0.39 versus 4.05 ± 0.98 , $P=0.027$) (Figure 3C and 3D). These findings indicate an early hyperemic response to inflammation that, through better oxygenation saturation and cell metabolism, results in preservation of the ischemic spinal cord tissue.

SWT Protects From Neuronal Degeneration in Ischemic Human Spinal Cords

To assess whether the effects found in the mouse model could be reproduced in human spinal tissue, we harvested spinal cord from a cadaver 2 hours postmortem, which was then defined as ischemia time. Spinal slice culture of the human samples was performed, and shock wave treatment was applied ex vivo. Fluoro-Jade B immunostaining for degenerating neurons revealed no difference between treated spinal slices and untreated controls 24 hours after shock wave application (CTR versus SWT: 14.67 ± 1.05 versus 16.40 ± 1.1 , $P=0.35$). The number of degenerating neurons remained stable in the treatment group, whereas the number increased significantly in controls at 48 hours after SWT (CTR versus SWT: 25.40 ± 1.28 versus 17.00 ± 1.98 , $P=0.032$), indicating that shock wave treatment protects from neuronal degeneration on ischemia (Figure 4A). Total cell count

revealed significantly higher numbers of cells in the treatment group at 24 hours (CTR versus SWT: 346.00 ± 36.14 versus 533.40 ± 30.85 , $P=0.017$) and 48 hours (CTR versus SWT: 293.40 ± 6.48 versus 532.20 ± 27.81 , $P=0.008$) after treatment. Higher numbers of cells in the treatment group could be due to either increased cell survival after shock wave treatment or cell infiltration.

Shock Wave Effect on Neuronal Survival Is TLR3 Dependent

In previous experiments, we found that the regenerative effect of shock waves on ischemic muscle is TLR3 dependent. To investigate whether the mechanism of action on ischemic spinal cords is the same, we again performed spinal slice cultures from wild-type and TLR3^{-/-} mice. We found significantly fewer degenerating neurons in the wild-type treatment group at 24 hours (CTR versus SWT: 73.40 ± 16.16 versus 35.15 ± 12.73 , $P=0.0022$) and 48 hours (CTR versus SWT: 85.87 ± 14.46 versus 40.85 ± 9.29 , $P<0.0001$) after shock wave application compared with untreated controls (Figure 5A). This effect was totally missing in TLR3^{-/-} mice (CTR versus SWT after 24 hours: 78.10 ± 16.85 versus 72.00 ± 21.81 , $P>0.05$; after 48 hours: 88.13 ± 19.52 versus 89.30 ± 20.60 , $P>0.05$), indicating that shock wave signaling is TLR3 dependent (Figure 5B).

It is well described that TLR4 is involved in neurodegeneration.^{12,14} It was also found that TLR4-dependent microglial activation mediates spinal cord ischemia–reperfusion injury.¹¹ TLR3 and TLR4 share a common signaling pathway via TRIF¹⁷; therefore, we sought to elucidate whether TLR4 was also stimulated by shock waves. Spinal slice culture from TLR4^{-/-} mice showed the same positive effect as in wild-type animals (CTR versus SWT after 24 hours: 84.70 ± 17.81 versus 38.80 ± 10.26 , $P=0.0095$; after 48 hours: 103.50 ± 16.09 versus 46.60 ± 11.84 , $P=0.0055$), showing that the effect is solely dependent on TLR3 and not TLR4 (Figure 5C). Western blot analysis confirmed an increase in TLR3 protein following SWT, whereas TLR4 protein was downregulated compared with untreated control spinal slices (Figure 5D). TLR4 is not unaffected by shock wave treatment, but it is downregulated. This finding is in line with and supports the earlier finding of TLR4 activation being responsible for mediating reperfusion injury and worsening outcome after spinal cord ischemia.¹¹

SWT Improves Functional Outcome and Survival

To investigate whether the observed effects on the cellular and subcellular levels led to functional improvement of treated animals, standardized motor tests were performed. Tests showed impressive functional improvement of the shock

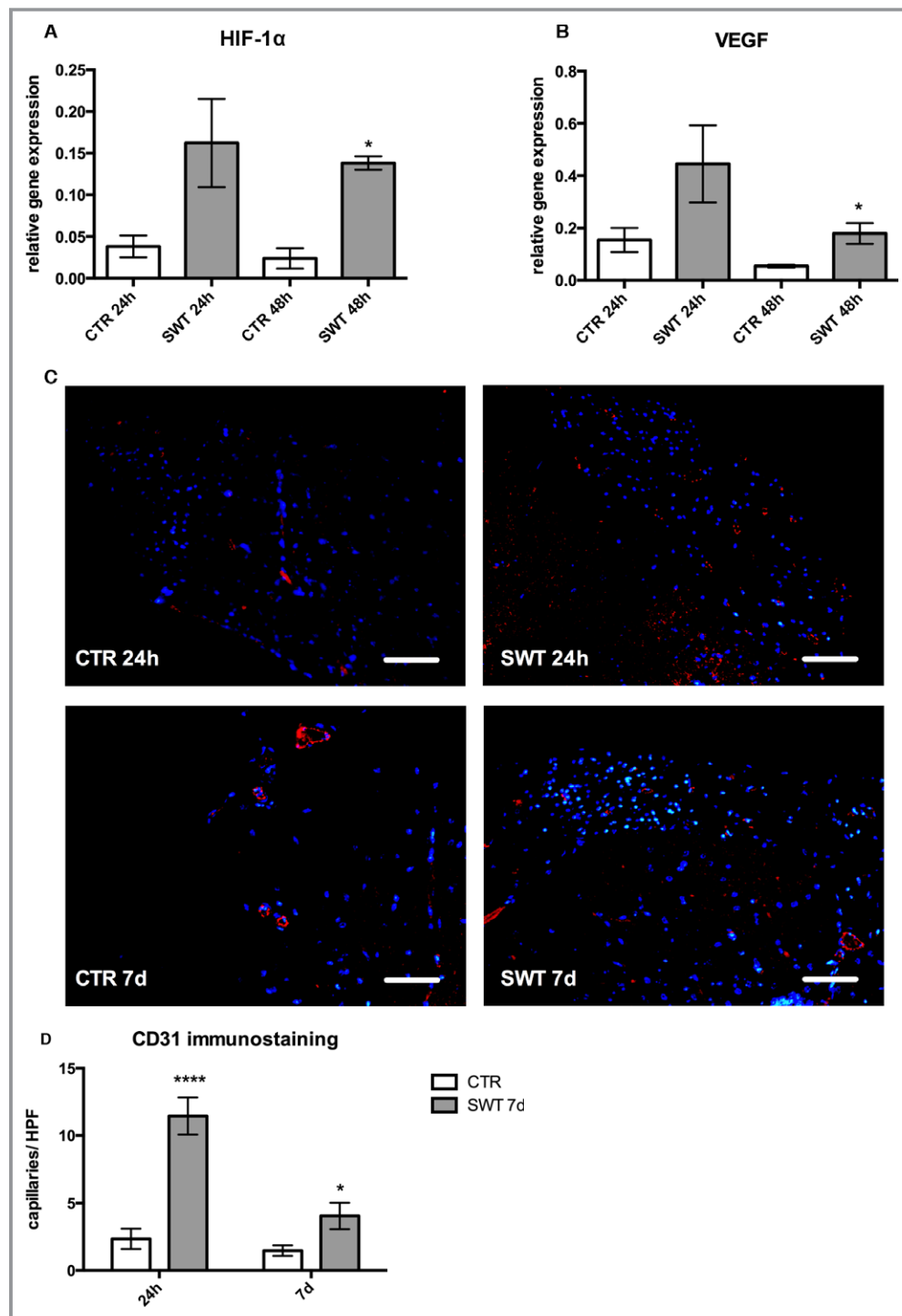


Figure 3. Shock wave therapy induces angiogenesis by capillary sprouting. A, The major proangiogenic player HIF-1 α was significantly increased in the treatment group at 24 and 48 hours after injury and treatment. $*P<0.05$. B, VEGF mRNA was found to be increased significantly 48 hours after treatment, indicating a strong angiogenic effect of shock wave treatment. $*P<0.05$. C, Representative pictures of CD31 immunostaining for capillaries showing increased capillary sprouting in the treatment group, enabling for better oxygen saturation and cell metabolism in the ischemic spinal cord tissue. DAPI was used for counterstaining of cell nuclei. Caliber represents 20 μm . D, Quantification of CD31 staining, given as capillaries per high-power field. $*P<0.05$, $****P<0.0001$. CTR indicates control; d, days; h, hours; HIF-1 α , hypoxia inducible factor 1 α ; HPF, high-power field; SWT, shock wave therapy; VEGF, vascular endothelial growth factor.

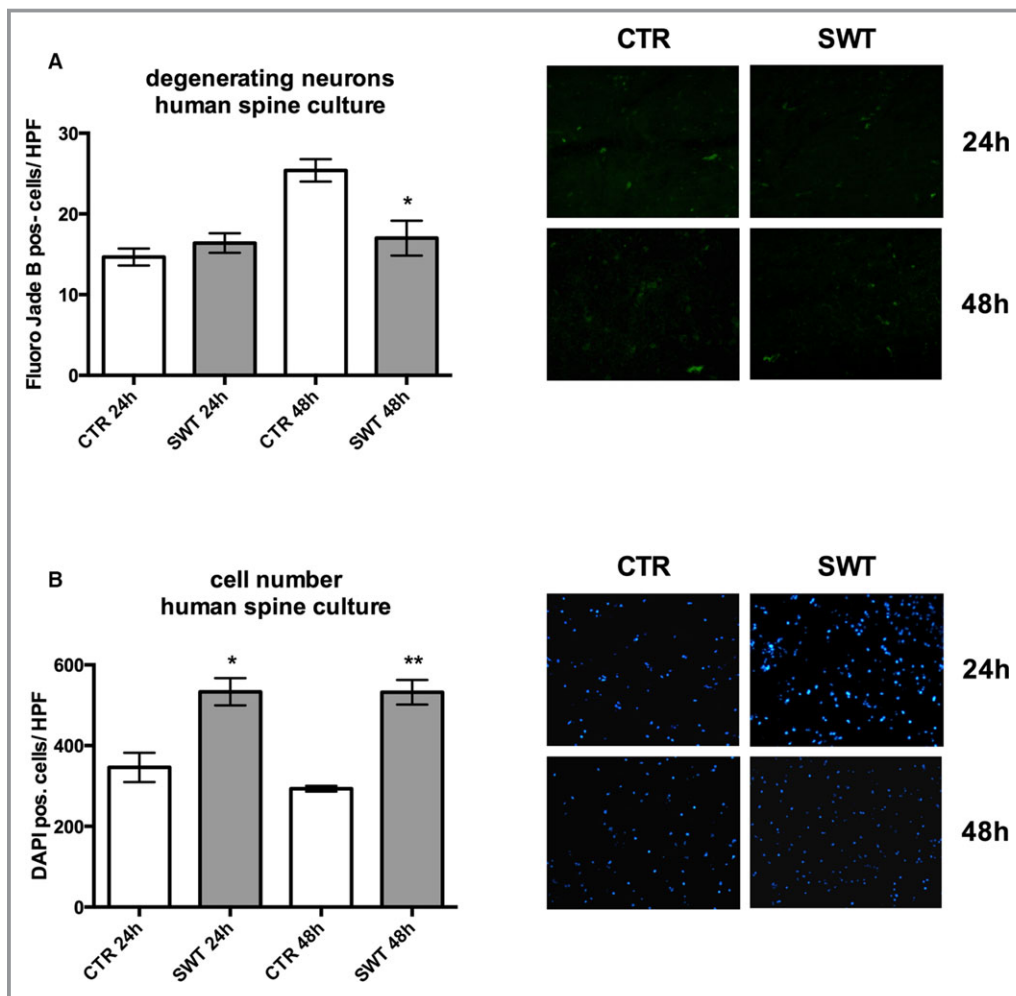


Figure 4. Shock wave therapy protects from neuronal degeneration in ischemic human spinal cords. A, Human spinal slice culture revealed a protective effect of shock wave therapy. Spinal cord was harvested from a cadaver 2 hours postmortem. At 48 hours after treatment, the number of degenerating neurons as shown by Fluoro-Jade B immunostaining was significantly lower in the treatment group, still on the level that it was before after 24 hours. In contrast, the number of degenerating neurons increased in untreated controls. This result in human tissue is in line with our findings from the in vivo mouse model (Figure 1B) and confirms that spinal shock wave therapy in patients would be effective. * $P < 0.05$. B, The total number of cells assessed by DAPI staining was significantly higher in shock wave-treated spinal slices compared with untreated controls at both 24 and 48 hours. * $P < 0.05$, ** $P < 0.01$. CTR indicates control; d, days; h, hours; SWT, shock wave therapy.

wave-treated animals compared with untreated controls. Improved performance was found in the cylinder test (upright position during 3 minutes for CTR versus SWT: day 1: 6.18 ± 2.49 versus 19.6 ± 1.03 ; day 3: 11.0 ± 1.0 versus 21.2 ± 0.73 ; day 7: 13.33 ± 2.4 versus 24.4 ± 1.63 , $P = 0.0171$) (Figure 6A), the locomotor test (fields per 30 seconds for CTR versus SWT: day 1: 7.33 ± 0.88 versus 17.25 ± 0.63 ; day 3: 11.0 ± 1.16 versus 21.0 ± 1.47 ; day 7: 12.33 ± 1.45 versus 22.25 ± 1.38 , $P = 0.0061$) (Figure 6B), the beam-walking test (seconds per beam walk for CTR versus SWT: day 1: 86.20 ± 17.30 versus 25.83 ± 2.33 ; day 3: 42.50 ± 7.50 versus 17.33 ± 1.20 ; day 7: 47.50 ± 2.50 versus 14.83 ± 1.42 ,

$P = 0.0786$) (Figure 6C), the beam-walking side slips (number of side slips for CTR versus SWT: day 1: 8.4 ± 1.54 versus 3.33 ± 0.42 ; day 3: 6.0 ± 1.0 versus 2.50 ± 0.50 ; day 7: 7.0 ± 1.0 versus 2.17 ± 0.48 , $P = 0.0751$) (Figure 6D), and the grip test (seconds of grip for CTR versus SWT: day 1: 69.83 ± 10.64 versus 181.0 ± 19.25 ; day 3: 130.0 ± 15.28 versus 238.0 ± 21.37 ; day 7: 146.7 ± 14.53 versus 299.2 ± 12.41 , $P = 0.0405$), although some results were not significant (Figure 6E).

Furthermore, we observed better survival after shock wave treatment compared with untreated controls. Treated animals that survived the first 24 hours all reached the study end

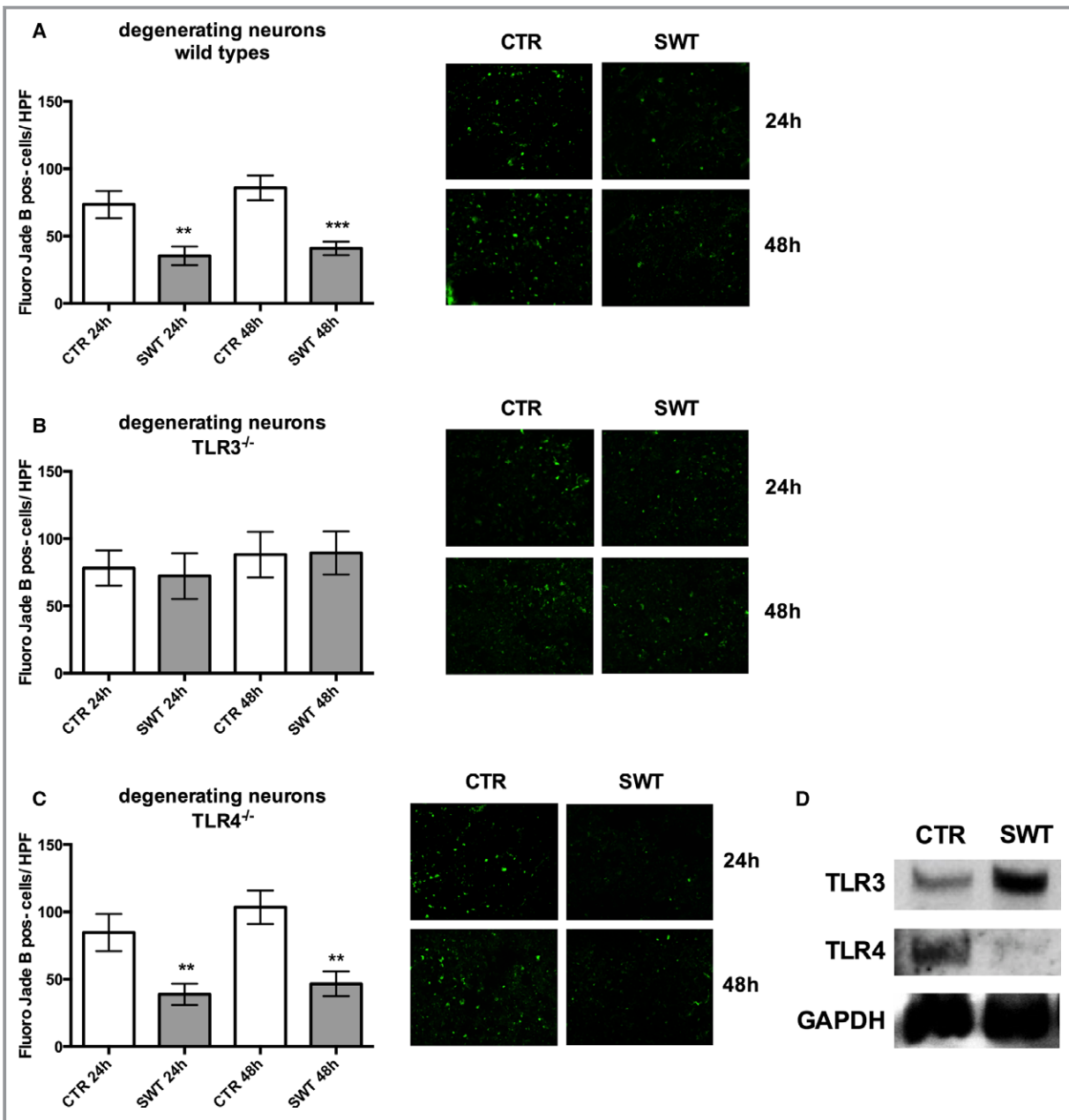
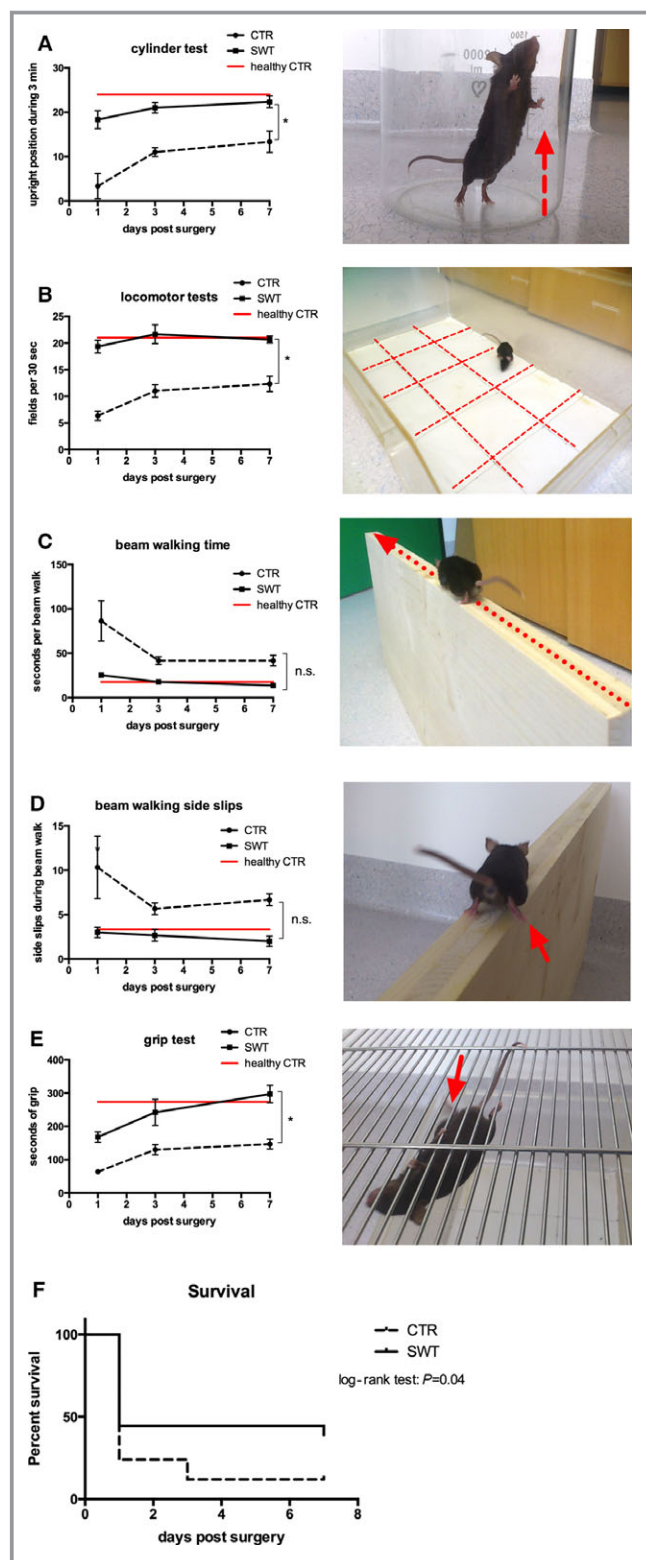


Figure 5. Shock wave effect on neuronal survival is TLR3 dependent. A, In vivo findings of significantly lower numbers of degenerating neurons, as quantified from Fluoro-Jade B immunostaining, could be reproduced in an ex vivo spinal slice culture from wild-type mice that was conducted to gain more insight into the working mechanism. $**P < 0.01$, $***P < 0.001$. B, In previously published experiments from ischemic muscle tissue, we found that the shock wave effect is strongly TLR3 dependent, thus we aimed to clarify whether the protective effect on neurons would be abolished in spinal cords from TLR3^{-/-} mice. Indeed, no effect could be observed compared with untreated controls, indicating that shock wave treatment depends on TLR3 signaling in spinal cord tissue. C, TLR3 and TLR4 are well known to share a common signaling pathway. Moreover, it was found that TLR4-dependent microglial activation mediates spinal cord ischemia–reperfusion injury; therefore, we wanted to show that the protective effect of shock waves was not diminished in TLR4^{-/-} mice. Spinal slice culture revealed effects comparable to those in wild types, showing fewer degenerating neurons in the treatment group at 24 and 48 hours after shock wave application. $**P < 0.01$. D, To prove TLR3 activation at a protein level, Western blot analysis was performed. It confirmed an increase in TLR3 protein following shock wave treatment, whereas TLR4 protein was downregulated compared with untreated control spinal slices. This finding indicates that the neuron-protective role of shock waves is a consequence of TLR3 activation and TLR4 downregulation in parallel. CTR indicates control; d, days; h, hours; HPF, high-power field; SWT, shock wave therapy, TLR3, Toll-like receptor 3; TLR4, Toll-like receptor 4.



point at day 7, whereas untreated controls had a worse survival rate (percentage survival for CTR versus SWT: day 1: 24% versus 44.4%; day 3: 12% versus 44%; day 7: 12% versus 38.9%; log-rank test $P=0.044$) (Figure 6F).

Figure 6. Shock wave therapy improves functional outcome and survival. A, In the so-called cylinder test, it was counted how often a mouse stood up on its hind limbs during 30 seconds as an indicator of functionality of the lower body. $*P<0.05$. B, The locomotor test counted the number of fields that a mouse crossed per 30 seconds and was another parameter for mobility, agility, and speed. $*P<0.05$. C, The beam-walking time was an indicator for speed and coordination. D, The number of slips during a beam walk was the main parameter for the measurement of coordination. Mice still showing paraparesis or even mild paraplegia slipped off the beam more often. E, The grip test measured the time that a mouse was able to hold itself in an inverted position and gave clear information about strength and functionality of the hind limbs. $*P<0.05$. F, The Kaplan–Meier curve showed that more than two-thirds of the untreated control animals did not survive the first 24 hours. This is due to severe visceral ischemia. A much higher percentage of the shock wave-treated animals survived the study end point of 7 days. CTR indicates control; min, minutes; sec, seconds; SWT, shock wave therapy.

Discussion

Having shown encouraging results in other ischemic conditions such as burn wounds and myocardial infarction, we aimed to investigate whether SWT would also be effective for the treatment of acute spinal cord ischemia. In recent work, we identified TLR3 signaling as mediating shock wave effects. TLR3 also causes downregulation of TLR4.¹⁷ TLR4 has been shown to trigger neuronal degeneration and to be involved in several kinds of neurological disorders.^{12,14,15} TLR3 activation with the synthetic RNA analogue poly(I:C) and simultaneous TLR4 downregulation have been described as protective against ischemia–reperfusion injury of the spinal cord.¹¹

SWT Ameliorates Motor Function and Increases Survival

Functional and clinical outcomes of our study were impressive and exceeded our expectations. Motor tests that measure mobility, coordination, strength, and speed of animals showed close to complete recovery of the treated animals as early as 3 days after treatment. Moreover, it was extremely challenging to make the control animals survive to the study end point of 7 days because aortic clamping caused severe visceral ischemia. Only 24% of the untreated animals survived the first 24 hours, whereas nearly twice as many of the treated animals survived. Shock wave treatment after spinal cord ischemia seemed to enhance early survival.

SWT Induces Angiogenesis and Modulates Inflammation via TLR3 Activation

Significantly higher numbers of capillaries were found in the treatment group compared with untreated controls. Accord-

ingly, the angiogenic factors VEGF and HIF-1 α were significantly increased. This proangiogenic effect was accompanied by a proinflammatory response to shock wave treatment, as described previously in the literature.^{21,24} Inflammation is an integral part of angiogenesis and was mediated by an early increase of IL-6 and transforming growth factor β mRNA expression that resulted in amplified macrophage recruitment. Activation of microglia that represent macrophages of the spinal cord was proven by Iba1 immunostaining and revealed a more stable condition of shock wave-treated animals over time compared with untreated controls. Microglia are a type of cell in the resting central nervous system that expresses TLR3 and TLR4. Although the mechanism has to be investigated in more detail in future experiments, it became evident that microglia play a crucial role by TLR3 activation in spinal cord protection or recovery after ischemia.

SWT Protects From Neuronal Degeneration

The observed effects led to improved neuronal survival after shock wave treatment, as shown by Fluoro-Jade B staining. Of note, the number of degenerating neurons in the treatment group remained stable over time. Neurons that were not damaged as early as 24 hours after injury were not injured later on, whereas neuronal degeneration could be shown to be progressive in untreated controls.

Data from animal experiments could be reproduced and confirmed in a human spinal slice culture, indicating that this treatment not only works in relatively young small animals without comorbidities but also exerts its beneficial effects in human tissue.

Neuronal Protection Is TLR3 Dependent

In ischemic muscle, we had identified the effect of shock waves as TLR3 dependent.¹⁷ In the current study, we were able to confirm this mechanism of action as being involved in spinal cord tissue. The effect of neuronal protection was almost completely abolished in TLR3-knockout mice, indicating the pivotal role of TLR3 signaling on SWT. TLR3 shares a common signaling pathway with TLR4; therefore, we performed the same experiments in a spinal slice culture from TLR4-knockout mice. Effects were as strong as in wild types, showing that shock waves are not TLR4 dependent; however, we observed a decrease of TLR4 protein in wild types. TLR4 had been described as being involved in the pathogenesis of several kinds of neurological disorders.¹⁴ The main mechanism of action of shock waves in spinal cord ischemia is apparently the same as in ischemic muscle tissue, namely, TLR3 stimulation and subsequent TLR4 downregulation.

Conclusion

In summary, shock wave treatment after spinal cord ischemia results in highly improved functional and clinical outcome. As an underlying mechanism, increased neuronal survival by modulated inflammation via TLR3 signaling, and thus induction of angiogenesis, could be demonstrated. Moreover, downregulation of TLR4 seems to have a protective effect in parallel.

Because SWT has already been proven safe in numerous clinical settings, the translation to clinical use should not be difficult. Because paraplegia and paraparesis due to spinal cord ischemia after surgical or endovascular aortic repair are exceptional entities, enrolling patients into a clinical study will be challenging and will require a multicenter approach. Nevertheless, SWT should be further investigated as a very rare regenerative strategy that has proven feasible and effective for the treatment of spinal cord ischemia.

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Disclosures

None.

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