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Extracorporeal Shock Wave Therapy Accelerates Regeneration After Acute Skeletal Muscle Injury

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Background: Muscle injuries are among the most common sports-related lesions in athletes; however, optimal treatment remains obscure. Extracorporeal shock wave therapy (ESWT) may be a promising approach in this context, because it has gained increasing importance in tissue regeneration in various medical fields.

Hypothesis: ESWT stimulates and accelerates regenerative processes of acute muscle injuries.

Study Design: Controlled laboratory study.

Methods: Adult Sprague-Dawley rats were divided into 4 experimental groups (2 ESWT+ groups and 2 ESWT– groups) as well as an uninjured control group ($n \geq 6$ in each group). An acute cardiotoxin-induced injury was set into the quadriceps femoris muscle of rats in the experimental groups. A single ESWT session was administered to injured muscles of the ESWT+ groups 1 day after injury, whereas ESWT– groups received no further treatment. At 4 and 7 days after injury, 1 each of the ESWT+ and ESWT– groups was euthanized. Regenerating lesions were excised and analyzed by histomorphometry and immunohistochemistry to assess fiber size, myonuclear content, and recruitment of satellite cells.

Results: The size and myonuclear content of regenerating fibers in ESWT+ muscle was significantly increased compared with ESWT– muscle fibers at both 4 and 7 days after injury. Similarly, at both time points, ESWT+ muscles exhibited significantly higher contents of pax7-positive satellite cells, mitotically active $H3P^+$ cells, and, of cells expressing the myogenic regulatory factors, myoD and myogenin, indicating enhanced proliferation and differentiation rates of satellite cells after ESWT. Mitotic activity at 4 days after injury was doubled in ESWT+ compared with ESWT– muscles.

Conclusion: ESWT stimulates regeneration of skeletal muscle tissue and accelerates repair processes.

Clinical Relevance: We provide evidence for accelerated regeneration of damaged skeletal muscle after ESWT. Although further studies are necessary, our findings support the view that ESWT is an effective method to improve muscle healing, with special relevance to sports injuries.

Keywords: muscle regeneration; molecular biology; healing enhancements

Mammalian skeletal muscle exhibits a remarkable ability to adapt to physiological stressors such as exercise and mechanical damage. The regenerative capacity of skeletal muscle tissue is of particularly high relevance in competitive sports, because muscle lesions are among the most common injuries in athletes.[23,47] Sports-related muscle damage is frequently associated with recurrence and can involve chronic pain, dysfunction, and even compartment syndrome,[12] which can delay or hinder an individual's training or participation in competition for several weeks.[47] Although this generates a high demand for treatment, skeletal muscle injuries often present a challenging problem to therapists because damaged muscle heals slowly and often with incomplete functional recovery,[21] and therapies available in current clinical practice often prove unsatisfying.[3,12,23] Early intervention to control the

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inflammatory response is shown to have ambiguous effects (with nonsteroidal anti-inflammatory drugs)[48] or even delayed or irreversibly disturbed healing (with corticosteroids).[4] Growth factor–mediated treatment by intramuscular injection of autologous platelet-rich plasma has been found ineffective in randomized double-blind, placebo-controlled clinical studies[38] and is associated with the caveats of unknown side effects. Accelerative effects on muscle repair have been found for low-level laser therapy and hyperbaric oxygen therapy, acting by upregulation of myogenic regulatory factor expression, including pax7, myoD, and myogenin.[5,19] However, both techniques remain controversial among clinicians.

Extracorporeal shock wave therapy (ESWT) may be a promising alternative approach in this context. The history of ESWT use in medicine dates back >40 years and developed in a multidisciplinary way.[18] Today, it is prominently used for lithotripsy and also to treat delayed and/or chronic wound healing,[32] coronary artery disease,[45] erectile dysfunction,[42] and musculoskeletal disorders.[22] However, despite considerable research efforts also utilizing animal models, the cellular and molecular mechanisms of ESWT still remain largely unclear. Previous work in rats has indicated that shock wave–enhanced tenocyte and bone regeneration is associated with increased expression levels of growth factors, including transforming growth factor–beta 1 and insulin-like growth factor 1.[8,43] Data from rats and human cell cultures indicate that ESWT upregulates the proliferation rates of mesenchymal stem cells,[8] smooth and cardiac muscle cells,[35] and endothelial precursor cells.[1] All of this substantiates the idea that ESWT affects muscle repair in similar ways, especially via influence on the muscle's main population of reserve cells, the so-called satellite cells.

It is generally accepted that skeletal muscle repair commences with a degradation process concomitant with an inflammatory phase, followed by an extensive repair phase and finally a maturation and remodeling phase.[21] The regenerative capacity of skeletal muscle depends mainly on satellite cells.[39] In response to muscle damage, these quiescent precursor cells become activated, proliferate, and enter differentiation to generate new muscle fibers. The signaling factors upregulated after ESWT are largely the same as those known to promote satellite cell activation after muscle damage.[7,10,36] Thus, we hypothesized that stimulation of the self-healing capacity via acceleration of signaling factor expression is a main mechanism of promotion of skeletal muscle repair in response to ESWT. The present study used immunohistology and digital histomorphometry to investigate the effects of a single ESWT session on satellite cell recruitment and growth of regenerating muscle fibers in an animal (rat) model of cardiotoxin (CTX)–induced muscle damage.

METHODS

Animals and Experimental Design

Experiments were performed on male Sprague-Dawley rats aged 10 to 12 weeks. Animals were housed in standard cages in a temperature-controlled room (22° ± 2°C) with a 12-hour/12-hour light/dark cycle and were fed pellets and tap water ad libitum. All experimental procedures were performed in accordance with the animal experiment guidelines issued by the Austrian Federal Ministry of Science, Research, and Economy.

Animals were divided into 4 experimental groups and 1 control group ($n \geq 6$ in each group). Rats in the experimental groups were anesthetized with isoflurane, and an acute muscle injury was set by injecting 500 μL of 10 μM CTX (from *Naja mossambica mossambica*; Sigma-Aldrich) in phosphate-buffered saline (PBS) into the left hindlimb (quadriceps femoris muscle; procedure of CTX injury modified after Couteaux et al[11]). Trypan blue (0.5%; Sigma Aldrich) was added to the PBS/CTX solution to enable macroscopic localization of injured muscle tissue. On postinjury day 1, animals in 2 of the experimental groups (ESWT+ groups) were anesthetized with isoflurane and received a single session of ESWT administered to the damaged hindlimb using a DuolithSD1 shock wave therapy device (Storz Medical). Each rat received a total of 500 impulses distributed to 5 spots along the longitudinal axis of the muscle (100 impulses/spot; energy flux density of 0.12 mJ/mm^2, 4 Hz). This dose lies within the range of doses currently used in human injury treatment.[33,37] The 2 remaining experimental groups received no treatment (ESWT– groups). Animals from 1 ESWT+ group and 1 ESWT– group were euthanized on postinjury day 4 (ESWT+/4d, ESWT–/4d) and day 7 (ESWT+/7d, ESWT–/7d), respectively. Trypan blue–stained areas of muscles were carefully excised, and muscle samples (1 × 1 × 5 mm) were fixed for further analysis. All samples taken from the ESWT+ and ESWT– groups were assigned an anonymized number-letter code to blind the treatment groups, thus reducing the risk of observer-based bias during histological evaluation. Rats in the control group were euthanized and sampled with the same method as above to provide reference values for the variables of interest in uninjured and untreated muscle.

All photographs were taken on a Reichert-Jung Polyvar microscope equipped for fluorescence microscopy using an Olympus digital camera system.

Histomorphometric Analysis

Samples intended for histological analysis were fixed in a solution of 2% paraformaldehyde (PFA) and 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer. Specimens were postfixed in 1% osmium tetroxide in 0.15 M sodium cacodylate buffer and embedded in glycid ether epoxy resin (Serva). Semithin cross sections (1.5 μm) were cut through the muscle lesions on a Reichert Ultracut S microtome (Leica Microsystems). Sections were deresinated with sodium methylate and stained with azure II methylene blue.

Three sections per lesion (minimum distance, 1 mm) were used for quantitative evaluation. On each section, a series of nonoverlapping photographs of the lesion center (3-4 depending upon lesion size) was taken using a ×40 oil immersion objective. Regenerating fibers to be included in the measurement were identified by their centrally positioned nuclei.[16] Photographs were superimposed with

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horizontal line grids with a spacing of 30 μm using ImageJ software (National Institutes of Health). Grid lines were used as count paths in the direction from the upper left to lower right. All regenerating fibers touched by grid lines were measured until they reached a total of 50 fibers per section, giving a total of 150 fibers from 3 sections per lesion/animal.

For each muscle fiber, the following variables were evaluated: cross-sectional area (CSA), minimal Feret diameter (F_{min}), and myonuclear content (number of central, peripheral, and total nuclei per fiber). Data from CSA measurements and the total number of nuclei per fiber served to determine CSA/myonuclei ratios.[25,40] Fiber size distributions were calculated using the F_{min} data. All measurements of length and area were performed using ImageJ software (version 1.50b).

Immunohistochemistry

Specimens intended for immunocytochemistry were cryofixed in liquid nitrogen–cooled 2-methylbutane. Within the lesions, 10-μm cross sections were cut on a 1720 Leitz cryostat and stored in liquid nitrogen until further processing.

Before immunostaining, sections were fixed in 4% PFA in PBS. After washing, sections were blocked in phosphate-buffered saline containing 0.5% TWEEN 20 (PBT) containing 2% bovine serum albumin and 5% normal goat serum and incubated with following primary antibodies: mouse monoclonal anti-pax7 IgG (1:20; Developmental Studies Hybridoma Bank) was used to detect pax7 protein, a marker of satellite cells; mouse monoclonal anti-myoD (5.8a) IgG (1:100; Santa Cruz) and mouse monoclonal anti-myogenin (anti-F5D) IgG (1:100; Developmental Studies Hybridoma Bank) were used to detect markers of myogenic determination (myoD) and early myogenic differentiation (myogenin), respectively; and rabbit polyclonal anti-phosphohistone H3 (Ser10) IgG (H3P) (1:200; Merck-Millipore) was employed to trace cells in mitotic proliferation. Alexa Fluor 546–labeled donkey antimouse IgG and Alexa Fluor 488–labeled goat anti-rabbit IgG served as secondary antibodies. All molecular markers analyzed are well established in research on muscle development and regeneration. Nuclei were counterstained with Hoechst 33258.

Photosampling followed a scheme similar to that described for semithin sections above, using 3 sections per lesion/animal (minimum distance, 1 mm). A series of 8 nonoverlapping, equidistant photographs was taken of each section. Provision was made that each such series covered the entire extent of the lesion. At each motif photographed, fluorescence signals were captured in the same focus plane according to the following scheme. Motifs were exclusively chosen and focused using the Hoechst signal. This signal depicts the position of all nuclei within the motif and enables clear demarcation of lesions according to their accumulations of mononucleated cells. Photographs on the 488-nm and 546-nm excitation channels were taken after a respective change of filters without any further adjustment of motif and focus. Positive nuclei were identified on the superimposed fluorescence images by the colocalization of the respective signals (green, red) with the blue Hoechst signal. Numbers of nuclei stained for pax7, myoD, myogenin, and H3P, and total numbers of nuclei were counted. Percentages of positive muscle cells were calculated for each molecular marker. Analyses were performed using ImageJ software.

Statistical Analysis

Because the data did not satisfy the assumptions of parametric homoscedastic additive models (tests and visual checks for normal distribution and homogeneity of variance failed), differences in measured variables between experimental groups (ESWT+ vs ESWT–) were tested by nonparametric methods. Intergroup differences were tested by the 1-way rank-based analog to analysis of variance (ANOVA; Kruskal-Wallis), followed by all pairwise multiple comparisons by the Dunn method, using SPSS 23.0 software (IBM Corp). Main effects and interaction effects between independent variables (treatment, time point after lesion) and measured response variables were examined by nonparametric rank-based, 2-way ANOVA-type tests using the R package nparLD.[34] Significance levels were set at $P < .05$.

RESULTS

Qualitative Analysis of Muscle Regeneration

A morphological examination of muscle recovery in the ESWT+ and ESWT– groups revealed a succession of degenerative and regenerative changes (Figure 1). On postinjury day 4, regenerating muscle was characterized by a loosened fiber arrangement constituted by a mixture of large necrotic fibers and small regenerating fibers with central nuclei. Extracellular spaces between fibers were infiltrated by mononucleated cells. On postinjury day 7, large numbers of regenerating fibers indicated a dynamic progression of muscle repair. Fiber arrays were more compact but were still less tight than in uninjured control muscles. Regenerating fibers were dominant, with only few mononucleated cells interspersed between them. At both sampling times, peripheral regions of the lesions contained some muscle fibers with normal polygonal appearance. A qualitative examination showed no obvious difference between the ESWT+ and ESWT– groups.

Size of Regenerating Muscle Fibers

Analysis of fiber size by measurement of F_{min} and CSA served to assess the growth rate of regenerating fibers in the lesions (Table 1). On postinjury day 4, the F_{min} of such fibers was 20 ± 2 μm in the ESWT+ group but only 17 ± 1 μm in the ESWT– group (Figure 1 and Table 1). Similarly, on day 7, the F_{min} was 32 ± 1 μm for the ESWT+ group but 28 ± 2 μm for the ESWT– group. On both days 4 and 7, the difference in F_{min} between the ESWT+ and ESWT– groups was significant at $P < .001$ (Table 1). Uninjured control muscle fibers were clearly

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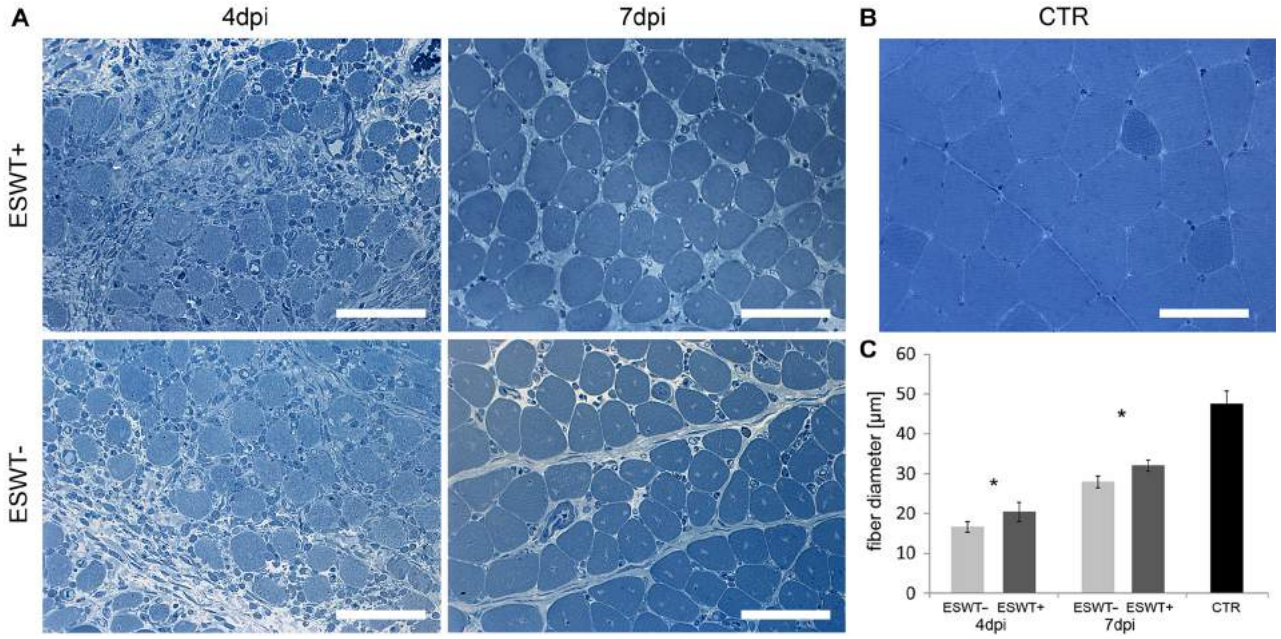

Figure 1. (A and B) Morphology of regenerating CTX-injured muscles and uninjured control muscle on semithin sections. Sections are as follows: top left, ESWT+ group euthanized on postinjury day 4 (ESWT+/4d); bottom left, ESWT– group euthanized on postinjury day 4 (ESWT–/4d); top middle, ESWT+ group euthanized on postinjury day 7 (ESWT+/7d); bottom middle, ESWT– group euthanized on postinjury day 7 (ESWT–/7d); and top right, control. On postinjury day 4, CTX muscles showed necrotic fibers and small regenerating fibers with central nuclei. Lesions were infiltrated by numerous mononucleated cells. On day 7, injured areas were dominated by regenerating fibers, with few mononucleated cells interspersed between them. Scale bars, 150 μm. (C) The minimal Feret diameter of regenerating fibers in ESWT+/4d, ESWT–/4d, ESWT+/7d, and ESWT–/7d groups and of uninjured CTR group fibers. *Statistically significant difference between ESWT+ and ESWT– groups ($P < .001$). 4dpi, 4 days postinjury; 7dpi, 7 days postinjury; CTR, control; CTX, cardiotoxin; ESWT+, group that received extracorporeal shock wave therapy; ESWT–, group that did not receive extracorporeal shock wave therapy.

larger, with an F_{min} of 48 ± 3 μm (Table 1) and a size range between 20 and 75 μm (Figure 2C). The mean F_{min} of ESWT+ muscles reached 42% and 67% of the control mean on days 4 and 7, respectively; corresponding ESWT– means were only at 35% and 58%, respectively. This is also reflected in the fiber size distributions (Figure 2, A and B): ESWT+ muscles contained more fibers >20 μm on day 4 and more fibers >30 μm on day 7. On day 4, ESWT+ muscle fibers additionally exhibited a broader size range compared with ESWT– fibers (5-45 vs 5-35 μm).

Results for fiber CSA were in accordance with those for F_{min}. ESWT+ muscle fibers were significantly larger than those of ESWT– muscles at both sampling times (Table 1).

Rank-based, 2-way ANOVA-type testing revealed a significant main effect of treatment on CSA ($P = .01$) but no interaction effects.

Myonuclear Content and CSA/Myonuclei Ratio of Regenerating Fibers

As an estimate of myoblast fusion to regenerating fibers, we used semithin cross sections to count myonuclei in regenerating fibers as identified by the presence of central nuclei. In fibers meeting this criterion, the vast majority of all nuclei is generally found in the centers (Table 2). Mean numbers of central nuclei, as well as mean numbers of peripheral and total nuclei, were significantly higher in the ESWT+ groups than in the ESWT– groups at both sampling times ($P \leq .03$) (Table 2). The total number of nuclei in the ESWT+ group exceeded that in the corresponding ESWT– group by >35% at each time. A higher content of nuclei in regenerating fibers after ESWT treatment was further confirmed by the finding that cross sections of most regenerating fibers of ESWT+ animals exhibited ≥3 nuclei, whereas those of ESWT– animal fibers mainly showed only 1 or 2 nuclei ($P < .001$ at both sampling times) (Table 2). The ESWT+/4d group also exhibited a significantly higher CSA/myonuclei ratio than the ESWT–/4d group. By contrast, there was no significant difference in the CSA/myonuclei ratio between the ESWT+/7d and ESWT–/7d groups (Table 2).

Rank-based, 2-way ANOVA-type testing showed a significant main effect of treatment on the number of nuclei at central positions (indicating recent uptake from fusing satellite cells[48]) and on total number of nuclei ($P < .001$ each).

Number of Satellite Cells

At each sampling time, the number of satellite cells, as identified by their pax7-positive ($pax7^+$) nuclei, was significantly

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TABLE 1
Size of Regenerating Fibers in the ESWT+, ESWT–, and Control Groups[a]

	Postinjury Day 4			Postinjury Day 7			
	ESWT+	ESWT–	*P* Value	ESWT+	ESWT–	*P* Value	Uninjured Controls
F_{min}, μm	20 ± 2	17 ± 1	<.001	32 ± 1	28 ± 2	<.001	48 ± 3
CSA, μm^2	477 ± 99	336 ± 50	<.001	1130 ± 90	890 ± 110	<.001	2450 ± 282

[a]Data are reported as means ± SD. Differences in F_{min} and CSA between ESWT+ and ESWT– groups on postinjury days 4 and 7 were statistically significant ($P < .001$). CSA, cross-sectional area; ESWT+, group that received extracorporeal shock wave therapy; ESWT–, group that did not receive extracorporeal shock wave therapy; F_{min}, minimal Feret diameter.

higher in ESWT+ muscle than in ESWT– muscle ($P = .02$ on day 4, $P < .001$ on day 7). On day 4, ESWT+ muscle contained 30% more $pax7^+$ nuclei than ESWT– muscle; until day 7, this difference grew to >50%. Satellite cell nuclei content expressed as a percentage of total nuclei was 3.7% ± 0.6% in the ESWT+/4d group, compared with only 2.8% ± 0.7% in the ESWT–/4d group, and 5.4% ± 0.5% in the ESWT+/7d group, compared with 3.5% ± 0.6% in the ESWT–/7d group (Figures 3 and 4). This finding implies that between postinjury days 4 and 7, the amount of $pax7^+$ cells increased by 46% in ESWT+ muscle but only by 25% in ESWT– muscle. For comparison, in untreated control muscle only 0.2% ± 0.2% of all nuclei were stained for pax7. The difference between shock wave–treated and untreated muscle is also reflected by the observation that the amount of $pax7^+$ satellite cells in ESWT+/4d muscle was rather similar to those in ESWT–/7d muscle and by a significant main effect of treatment on the number of $pax7^+$ cells, as shown by rank-based, 2-way ANOVA-type testing ($P < .001$).

Ratios of Proliferating Cells and Differentiating Myogenic Cells

Muscle lesions generally contain large numbers of mitotically active cells with $H3P^+$ nuclei (Figure 4). Particularly on postinjury day 4, the proportion of such nuclei was significantly higher in ESWT+ muscle than in ESWT– muscle (6.7% ± 1.1% vs 3.0% ± 0.6% of all nuclei; $P < .001$) (Figure 3), representing a 123% increase in the ESWT+/4d group. This difference in proliferating cell numbers was still present on day 7, although it was no longer statistically significant (8.2% ± 1.6% of all nuclei in the ESWT+/7d group vs 7.0% ± 1.4% in the ESWT–/7d group). Few or no $H3P^+$ nuclei were found in the control muscles (0.1% ± 0.1%).

Numbers of nuclei stained for the myogenic markers myoD and myogenin were determined to assess whether ESWT also affects the commitment of satellite cells to differentiate into new muscle fibers (Figure 4). At both time points, ESWT+ muscle contained significantly more $myoD^+$ nuclei compared with ESWT– muscle ($P < .001$ for both time points) (Figure 3). The most prominent difference was again found on day 4, at which time the percentage of $myoD^+$ nuclei in ESWT+ muscle exceeded that in ESWT– muscle by 125% (3.6% ± 0.5% vs 1.6% ± 0.2% of all nuclei). The corresponding ratios on day 7 were 5.1% ± 0.9% and 3.2% ± 1.0% for the ESWT+/7d and ESWT–/7d groups,

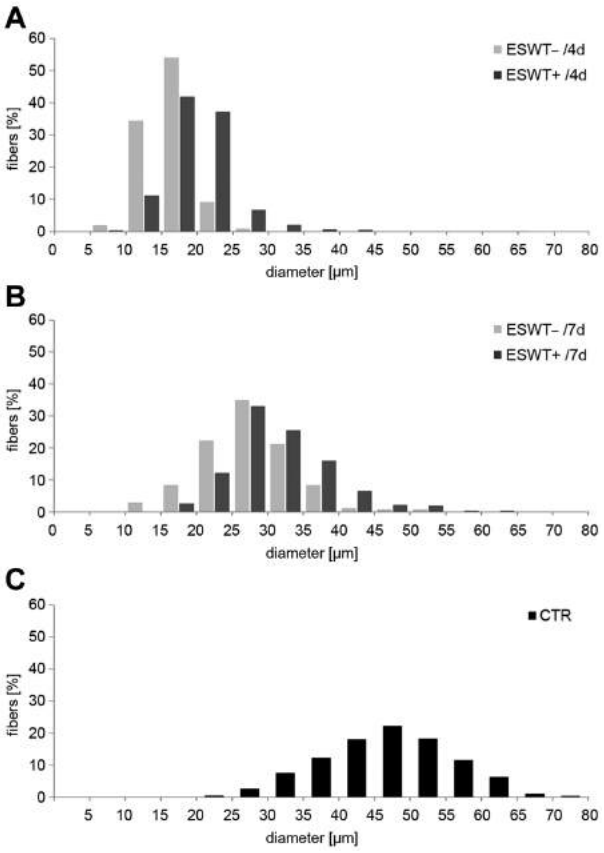

Figure 2. Fiber size distributions of ESWT+ and ESWT– muscles on (A) postinjury day 4, (B) day 7, and (C) control muscle. The F_{min} of 150 muscle fibers per animal are grouped in size classes of 5 μm, and fiber numbers in each case are plotted as percentage of the total count. ESWT+ groups exhibited larger fibers than ESWT– groups. CTR, control; ESWT+, group that received extracorporeal shock wave therapy; ESWT–, group that did not receive extracorporeal shock wave therapy.

respectively. No $myoD^+$ nuclei were found in control muscles. Similarly, percentages of $myogenin^+$ nuclei in ESWT+ muscle were significantly higher than in ESWT– muscle at both sampling times ($P < .001$ at each time point). Specifically, 2.8% ± 0.4% of all nuclei in ESWT+/4d muscle stained for myogenin compared with only 1.5% ± 0.3% in

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TABLE 2
Myonuclear Content and CSA/Myonuclei Ratio of Regenerating Fibers in ESWT+ and ESWT– Muscle[a]

	Postinjury Day 4			Postinjury Day 7		
	ESWT+	ESWT–	*P* Value	ESWT+	ESWT–	*P* Value
No. of nuclei/cross-sectioned fiber	2.6 ± 0.2	1.9 ± 0.2	<.001	2.6 ± 0.4	1.9 ± 0.1	<.001
No. of central nuclei/cross-sectioned fiber	2.2 ± 0.2	1.7 ± 0.1	<.001	1.8 ± 0.2	1.4 ± 0.1	<.001
No. of peripheral nuclei/cross-sectioned fiber	0.4 ± 0.1	0.1 ± 0.6	.03	0.8 ± 0.2	0.6 ± 0.1	<.001
<2 nuclei/fiber, %	54.8	75.4	<.001	53.5	77.4	<.001
≥3 nuclei/fiber, %	45.2	24.6	<.001	46.5	22.6	.001
CSA/myonuclei ratio, μm^2	241 ± 83	206 ± 26	.01	547 ± 90	536 ± 56	NS

[a]Data are reported as means ± SD unless otherwise indicated. CSA, cross-sectional area; ESWT+, group that received extracorporeal shock wave therapy; ESWT–, group that did not receive extracorporeal shock wave therapy; NS, not significant.

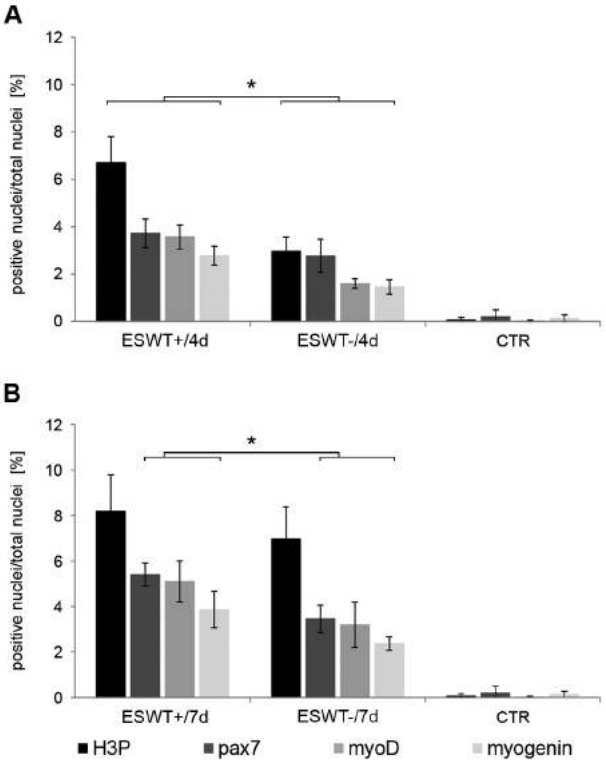

Figure 3. Mean (±SD) percentages of $H3P^+$, $pax7^+$, $myoD^+$, and $myogenin^+$ nuclei in ESWT+ and ESWT– muscles on (A) postinjury day 4, (B) on postinjury day 7, and in the control group. *Statistically significant difference between the ESWT+ and ESWT– groups ($P < .001$). CTR, control; ESWT+, group that received extracorporeal shock wave therapy; ESWT–, group that did not receive extracorporeal shock wave therapy.

the ESWT–/4d muscle and 3.9% ± 0.8% in ESWT+/7d muscle compared with only 2.4% ± 0.3% in ESWT–/7d muscle (Figure 3), suggesting enhanced differentiation of myogenic cells after ESWT. By contrast, control muscles contained only 0.1% ± 0.1% $myogenin^+$ nuclei (Figure 3).

As with $pax7^+$ above, results for H3P, myoD, and myogenin demonstrated that numbers of stained nuclei in ESWT+/4d muscle were similar to those in ESWT–/7d muscle.

Double labeling for H3P and myoD, intended for identification of myogenic cells in transition between proliferation and differentiation, revealed that numbers of $H3P^+myoD^+$ cells were low in all groups, with a trend to be higher in ESWT+ muscle than in ESWT– muscle (0.3% ± 0.1% vs 0.2% ± 0.1% of all nuclei on day 4, and 0.6% ± 0.3% vs 0.4% ± 0.3% on day 7; differences not significant).

Rank-based, 2-way ANOVA-type testing revealed significant main effects of treatment on all measured marker variables ($H3P^+$, $myoD^+$, $myogenin^+$, and $H3P^+myoD^+$; $P <$ .01 each).

DISCUSSION

Experimental modeling of skeletal muscle injury is a multifaceted task. Muscle injuries are commonly divided into an in situ necrosis type and a shearing type. In situ necrosis injuries are characterized by necrotized muscle fibers but intact basal laminae, whereas basal laminae, blood vessels, and mysial sheets are also impaired in shearing-type injuries, depending on severity.[23,27] CTX-induced myolysis as applied in the present study induces a reproducible necrosis-regeneration cycle[6] while leaving blood vessels and major parts of the basal laminae intact.[17] It thus preserves the classic satellite cell niche and likely provides a suitable model to investigate satellite cell behavior after necrosis-type injury. However, because the phases of repair have been found to be similar between the different types of muscle injury,[24] it is not at all unlikely that the CTX model is also valid for shearing-type injuries. In agreement with this, present therapies are already mainly selected according to the severity of the muscle injury rather than according to type.[24]

The results of the present study provide a clear indication that ESWT is able to accelerate the time course of regeneration in rat skeletal muscle after acute CTX-induced injury. Our results suggest that shock wave treatment applied shortly after muscle damage induces measurable effects on cellular repair mechanisms during both the initiation and maturation phases of the regeneration process.

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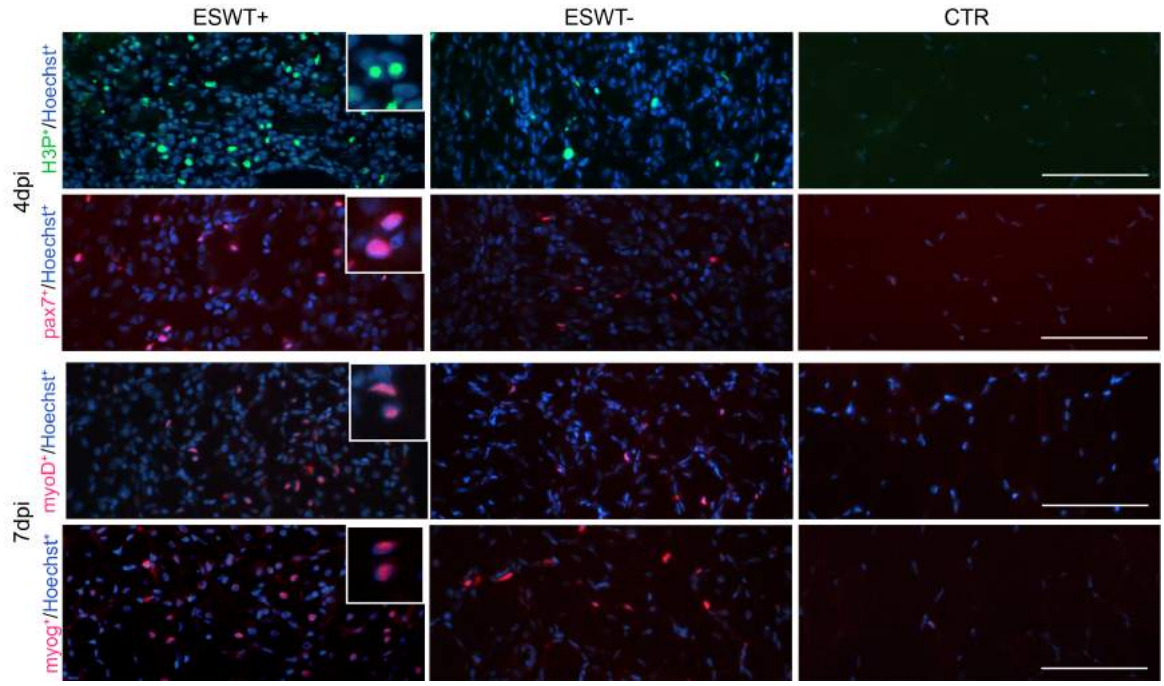

Figure 4. Fluorescence microscopy images of sections of ESWT+, ESWT–, and uninjured control muscles immunostained for H3P, Pax7, myoD, and myogenin. Nuclei counterstained with Hoechst 33258. Insets highlight colocalization of immunostaining signals and blue DNA stain in positive nuclei. Scale bars, 100 μm. 4dpi, 4 days postinjury; 7dpi, 7 days postinjury; CTR, control; ESWT+, group that received extracorporeal shock wave therapy; ESWT–, group that did not receive extracorporeal shock wave therapy.

These effects involve an increase in the amount of satellite cells recruited to the lesion site, accompanied and followed by an enhancement of proliferation and differentiation rates of these cells, as well as a faster growth of the newly formed muscle fibers. All molecular marker variables (Figure 3 and Table 3) showed that ESWT– muscles require 7 days to reach the level of regeneration that ESWT+ muscles have already achieved on postinjury day 4.

Although ESWT has successfully been implemented to treat soft tissue injuries,[18] this is to our knowledge the first investigation providing detailed information about how ESWT affects skeletal muscle regeneration. Only a few studies to date have addressed the effects on regeneration time, and until about a decade ago, it was concluded that there would be no significant effects on fiber size, myonuclear content, and myotube production.[2,46] The present work provides evidence that a single treatment is already sufficient to induce an effect that is initiated immediately after injury when Pax7-expressing satellite cells must be recruited into the lesion. This is regarded as indispensable for commencing adult skeletal muscle repair.[29,39] Our results document an increased presence of $Pax7^+$ satellite cells in CTX-induced lesions after ESWT (Figure 3 and Table 3). Numerous in vivo studies have shown that muscle injury induces the activation of satellite cells, which then proliferate and rapidly enter differentiation.[6] This is accompanied by upregulation of myoD, a primary determining factor of the muscle lineage present during activation and proliferation,[39] and of myogenin, which is expressed during satellite cell differentiation and fusion to multinucleated muscle fibers.[6] Measured numbers of $H3P^+$, $myoD^+$, and $myogenin^+$ cells (Figure 3 and Table 3) indicate that ESWT accelerates the time course of the regenerative cascade from satellite cell activation to fiber neogenesis. The increased presence of $H3P^+myoD^+$ cells in ESWT+ specimens on postinjury days 4 and 7 is in agreement with and further supports this interpretation, because it is known that myoD and other markers of myogenic commitment are expressed in myogenic cells that still undergo mitotic division.[26]

The pattern of ESWT influence on muscle regeneration inferred from our results is similar to that established for the effects of ESWT on various other cell types and tissues. This refers, for example, to enhanced osteoblast recruitment via mesenchymal stem cell activation in segmental defects of rat bone,[8] accelerated rat tenocyte differentiation after collagenase-induced Achilles tendinitis,[7] enhanced recruitment of endothelial progenitor cells via improved expression of chemoattractant factors in rat hindlimb ischemia,[1] and activation of human cultured primitive cardiomyocytes and endothelial and smooth muscle precursors.[35]

An accelerating effect of ESWT in the maturation phase of muscle regeneration is also evident from the larger size and higher myonuclear content of regenerating muscle fibers in the ESWT+ groups (Tables 1 and 2). Faster hypertrophic growth may result from a higher rate of myoblast fusion to regenerating fibers.[13] In the final stage of the regeneration, ESWT therefore likely mediates that the new fibers formed during the repair grow to mature size more rapidly, leading to full functional recovery in a shorter period of time. The additional finding that CSA/myonuclei

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TABLE 3
Total Numbers of Hoechst-stained Nuclei and Numbers and Percentages of Nuclei Immunostained for Molecular Markers[a]

	ESWT+			ESWT–		
	Total Nuclei	Positive Nuclei	Positive Nuclei/ Total Nuclei, %	Total Nuclei	Positive Nuclei	Positive Nuclei/ Total Nuclei, %
Day 4						
pax7$^+$	72,289	2560	3.7 ± 0.6	63,694	1681	2.8 ± 0.7
myoD$^+$	56,517	1789	3.6 ± 0.5	63,558	985	1.6 ± 0.2
myogenin$^+$	71,632	1840	2.8 ± 0.4	65,682	889	1.5 ± 0.3
H3P$^+$	72,289	4340	6.7 ± 1.1	63,694	1743	3.0 ± 0.6
H3P$^+$myoD$^+$	56,186	153	0.3 ± 0.1	63,229	88	0.2 ± 0.1
Day 7						
pax7$^+$	42,330	2240	5.4 ± 0.5	37,044	1262	3.5 ± 0.6
myoD$^+$	38,236	1898	5.1 ± 0.9	32,860	953	3.2 ± 1.0
myogenin$^+$	35,594	1381	3.9 ± 0.8	34,752	769	2.4 ± 0.3
H3P$^+$	42,330	3354	8.2 ± 1.6	37,044	2529	7.0 ± 1.4
H3P$^+$myoD$^+$	37,874	195	0.6 ± 0.3	32,669	97	0.4 ± 0.3

	Uninjured Controls		
	Total Nuclei	Positive Nuclei	Positive Nuclei/Total Nuclei, %
pax7$^+$	11,279	20	0.2 ± 0.2
myoD$^+$	11,924	1	0.0 ± 0.0
myogenin$^+$	11,425	13	0.1 ± 0.1
H3P$^+$	11,279	5	0.1 ± 0.1
H3P$^+$myoD$^+$	12,007	11	0.0 ± 0.0

[a]Values are reported as numbers or means ± SD. ESWT+, group that received extracorporeal shock wave therapy; ESWT–, group that did not receive extracorporeal shock wave therapy.

ratios are largely unaffected (Table 2) provides further indication that the enhanced growth of regenerating fibers in ESWT+ animals relies on efficient myonuclear supply.

Together, our findings provide a substantial indication that ESWT is able to accelerate skeletal muscle repair. Sports medicine is a promising field to be considered for application of ESWT in clinical practice because especially here, the fast and complete repair of muscle injuries is a primary goal.[23] In light of the presently unsatisfactory therapeutic situation,[4,38,50] ESWT lends itself as a promising noninvasive and conveniently applicable method to improve muscle healing after sports injuries, as well as an alternative to approaches such as platelet-rich plasma, low-level laser therapy, and hyperbaric oxygen therapy. Testing the method in clinical application is therefore considered worthwhile also with regard to possible anti-inflammatory, angiogenic, and analgesic effects. Important hints as to the existence of such effects derive from clinical studies of ESWT influence on cardiac neovascularization[44] and chronic joint and musculoskeletal pain.[28,30]

Although the present study provides evidence for a positive effect of ESWT on muscle repair, the exact mechanism of interaction between shock waves and tissue remains unclear. It has been postulated that the biological effect is based on mechanotransduction,[14] which results in specific gene expression patterns in target tissues.[15] Indeed, skeletal muscle is known to be highly responsive to mechanical stimulation,[41] resulting in muscle fiber growth mediated by increased protein synthesis and/or satellite cell recruitment.[20] In addition, shock waves have been shown to upregulate a variety of signaling factors relevant to regeneration, including nitric oxide, insulin-like growth factor, and fibroblast growth factor, in a variety of tissues.[7,9,31] It may thus be assumed that it is a complex combination of several factors by which shock waves act to stimulate muscle healing. Further research is required to clarify this aspect.

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