Received: 17 January 2019 | Accepted: 4 December 2019

DOI: 10.1002/jor.24564

Journal of Orthopaedic Research®

RESEARCH ARTICLE

Exposure to radial extracorporeal shockwaves induces muscle regeneration after muscle injury in a surgical rat model

Eva K. Langendorf | Anja Klein | Philipp Drees | Pol M. Rommens | Stefan G. Mattyasovszky | Ulrike Ritz

Department of Orthopedics and Traumatology, University Medical Centre of the Johannes Gutenberg-University of Mainz, Mainz, Germany

Correspondence
Stefan Mattyasovszky, Department of Orthopedics and Traumatology, University Medical Centre of the Johannes Gutenberg-University of Mainz, Langenbeckstraße 1, 55131 Mainz, Germany.
Email: stefan.mattyasovszky@gmx.de

Funding information
Internal funding of the University Medical Center of the Johannes Gutenberg-University of Mainz, Grant/Award Number: Stufe I

Abstract

The leading cause of training interruption in sport is a muscle injury, for which the standard treatment is nonsteroidal anti-inflammatory drugs (NSAIDs). To find alternative treatments, we investigated whether the radial extracorporeal shockwave application (rESWT) could stimulate muscle regeneration. A lesion with complete rupture (grade III muscle tear) was set in the musculus rectus femoris of 12-week-old Wistar rats, and the NSAID diclofenac, rESWT, or a combined therapy were applied on day 0, 3, and 5 directly following the surgery. Rats were euthanized at 2, 4, and 7 days after surgery and the area of muscle lesion was excised for histological and gene expression analysis to determine the progress in the healing of damaged fibers and tissue regeneration. The best effect on muscle regeneration was observed in the group treated with rESWT alone. Monotherapy by diclofenac showed a smaller but still positive effect and lowest effects were detected when both therapies were applied. rESWT alone demonstrated a significant upregulation of the muscle markers MyoD and myosin. The presence of myosin gene expression indicated newly formed muscle fibers, which was confirmed by hematoxylin and eosin staining. Seven days after injury the amount of mononucleated cell decreased and regenerating fibers could be detected. This effect is most pronounced in the group treated with rESWT alone. In our study, shockwaves demonstrated the best effect on muscle regeneration. Therefore, we recommend prospective clinical studies to analyze the effect of rESWT after sports trauma to improve muscle regeneration and to shorten the rehabilitation.

KEYWORDS
radial extracorporeal shockwaves, muscle regeneration, surgical in vivo model of muscle injury

1 | INTRODUCTION

The leading cause of training interruption and breaks from sporting competitions is a muscle injury. Depending on the type of sport, muscle injuries represent between 23% and 46% of all injuries.[1,2]

Stefan G. Mattyasovszky and Ulrike Ritz contributed equally to this study.

Normally, a conservative treatment consisting of cooling, rest, and relaxation is the therapy of choice. Additive therapies have been suggested to shorten the healing process and thereby accelerating the return to full activity.[3] Examples of such therapies are injections of steroids or anesthetics,[4] treatment with platelet-rich plasma[5] or antifibrotic agents,[6] injection of actovegin and traumeel,[7] or the use of stem cells.[8] However, there is a lack of agreement between physiotherapists, sports clinicians, and scientists on the use of these additional therapies.

Following injury, muscle-specific genes, as well as stem and satellite cells, are involved in the complex process of muscle regeneration.[9] Approximately 1 week after injury, activation, proliferation, and differentiation of muscle stem cells commences, and this is accompanied by the formation of new muscle fibers. Subsequently, reconstitution of the muscle takes place.[10]

Radial extracorporeal shockwave application (rESWT) represents a promising alternative therapy for muscle regeneration. Shockwaves have been applied for conservative treatment in musculoskeletal diseases for over 20 years, to support physiotherapy and pain management[11,12] in conditions such as plantar fasciitis,[13] tendinopathy, tennis elbow, or calcified shoulder.[14] A positive influence on human osteoblasts[15,16] as well as on human skeletal muscle cells[17] was observed. However, the underlying mechanisms of rESWT on muscle regeneration remain unclear.

The application of nonsteroidal anti-inflammatory drugs (NSAIDs) is a short-term method to treat muscle injuries.[18] A readily available NSAID, diclofenac, is one of the most frequently used drugs in the treatment of acute muscle injuries.[19] However, these drugs can be associated with undesirable side effects, such as gastrointestinal, heart, and kidney complications.[20,21]

A number of rodent models are available to investigate the effect of different therapies on muscle injury, such as freeze injury, barium chloride ($BaCl_2$) injection, notexin (NTX), surgical muscle destruction by a needle[22] or a scalpel,[11] cardiotoxin, and others.[23] Surgical muscle destruction appears to be the most comparable method to injuries occurring during sport.[22]

In this study, we employed a surgical injury rat model, using a scalpel to create a structural muscle lesion (grade III muscle tear) to analyze the effects of rESWT and diclofenac in mono- and combined therapies on muscle regeneration. The objectives of this study were to determine the best treatment for accelerating healing following acute structural muscle lesions and to investigate the mechanism by which shockwaves influence muscle regeneration.

2 | MATERIALS AND METHODS

2.1 | In vivo model and experimental procedure

Our study was approved by the local regional animal welfare committee Landesuntersuchungsamt Rheinland-Pfalz (23 177-07/G15-1-038).

Twelve-week-old male Wistar rats (Janvier, France) were divided into four groups, each of which was subdivided into three subgroups. Each subgroup consisted of 10 animals (Table 1). Rats were housed in standard cages with a 12-hour light-dark rhythm. Rats were sedated with isoflurane following a subcutaneously administered anesthesia containing midazolam (2 mg/kg), medetomidine (150 μg/kg), and fentanyl (5 μg/kg). A 1-cm skin incision was made at the right femur between the lateral femoral condyle and the greater trochanter. The lesion was marked at both ends with knots of a 4-0 Safil-suture to identify the exact position for shockwave application as well as for postmortem examination. The muscle destruction (grade III muscle tear) was performed with a scalpel at the musculus rectus femoris transverse to the fiber

TABLE 1 Different groups and treatment variations

Group	n	rESWT Day 0	rESWT Day 3	rESWT Day 5	Diclofenac Day 0	†
Control 1	10	–	–	–	–	Day 1
Control 2	10	–	–	–	–	Day 3
Control 3	10	–	–	–	–	Day 7
Diclo 1	10	–	–	–	+	Day 2
Diclo 2	10	–	–	–	+	Day 4
Diclo 3	10	–	–	–	+	Day 7
rESWT 1	10	+	–	–	–	Day 2
rESWT 2	10	+	+	–	–	Day 4
rESWT 3	10	+	+	+	–	Day 7
rESWT + Diclo 1	10	+	–	–	+	Day 2
rESWT + Diclo 2	10	+	+	–	+	Day 4
rESWT + Diclo 3	10	+	+	+	+	Day 7

Abbreviation: rESWT, radial extracorporeal shockwave application.
†, different time points of sacrifice.

orientation, with a depth and width of 3 mm. For the skin closure, a 4-0 Safil-filament was used (Figure 1).

Rats were sacrificed at various time points by inhalation of CO_2. The marked area of the muscle incision was carefully excised. Muscle tissue was either fixed in Roti Histofix-4.5% (Carl Roth GmbH, Karlsruhe, Germany) for histological analyses or stored in RNAlater solution (Invitrogen; Life Technologies; Thermo Fisher Scientific, Carlsbad, CA) for investigation of gene expression.

2.2 | Therapy schedule

Radial shockwave application was performed using the Swiss DolorClast Classic (E.M.S., Nyon, Switzerland) under anesthesia at days 0, 3, and 5, with the following settings: 500 impulses, a frequency of 10 Hz, and a pressure of 2 bar, employing a shockwave applicator with a diameter of 15 mm. These settings were comparable to our in vitro experiments.[17] Diclofenac was applied subcutaneously in the neck fold directly after surgery, at a concentration of 2 g/kg body weight as previously published by Tomazoni et al.[24] The control group received no treatment. The rats were sacrificed as outlined in Table 1. In contrast to the therapy groups (sacrificed on days 2, 4, and 7), the control groups were euthanized on days 1, 3, and 7. This discrepancy was due to the fact that to reduce animal numbers, the control group data were derived from another study using the same model (see Section 4.4).

Our focus was to determine how many treatments were required, and how many days it took before any effects of shockwave therapy were observed in rats. Therefore, we treated one group with shockwaves once, directly after surgery, and sacrificed the animals in this group 2 days later. The second group received the first treatment directly after surgery and a second treatment on day 3 and were culled on day 4. The last group was treated directly after surgery, a second time on day 3 and for a third time on day 5 and were culled after 7 days (Table 1). The muscle tissue derived from five animals in each group was used for RNA isolation, while tissue from the other five animals was used for histological and immunohistochemical analyses.

2.3 | RNA isolation and gene expression analyses

Muscle tissue that lay between the two marking knots was excised and used for RNA isolation. Twenty milligrams of each sample tissue was lysed and homogenized using the Precellys Tissue RNA Kit (Peqlab, Erlangen, Germany). Isolation and purification of total RNA were performed using the kit RNA binding columns.

Reverse transcription was performed using 2 μg RNA, Superscript III Reverse transcriptase (Invitrogen; Life Technologies; Thermo Fisher Scientific), Random Primers (Promega, Madison, WI), and dNTPs (Bioron GmbH, Ludwigshafen, Germany). The gene expression analyses for 18S, Neural cell adhesion molecule (NCAM), Myf5, Myosin, and MyoD were performed using quantitative reverse transcription-polymerase chain reaction (PCR) with QuantiTect Primer Assays using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). The comparative threshold cycle (C_t) method was used to calculate the relative gene expression and presented as $2^{-\Delta\Delta C_t}$ values.[25] The gene expression was normalized to 18S rRNA. All samples were examined in triplicates (five different samples in triplicates, n = 15).

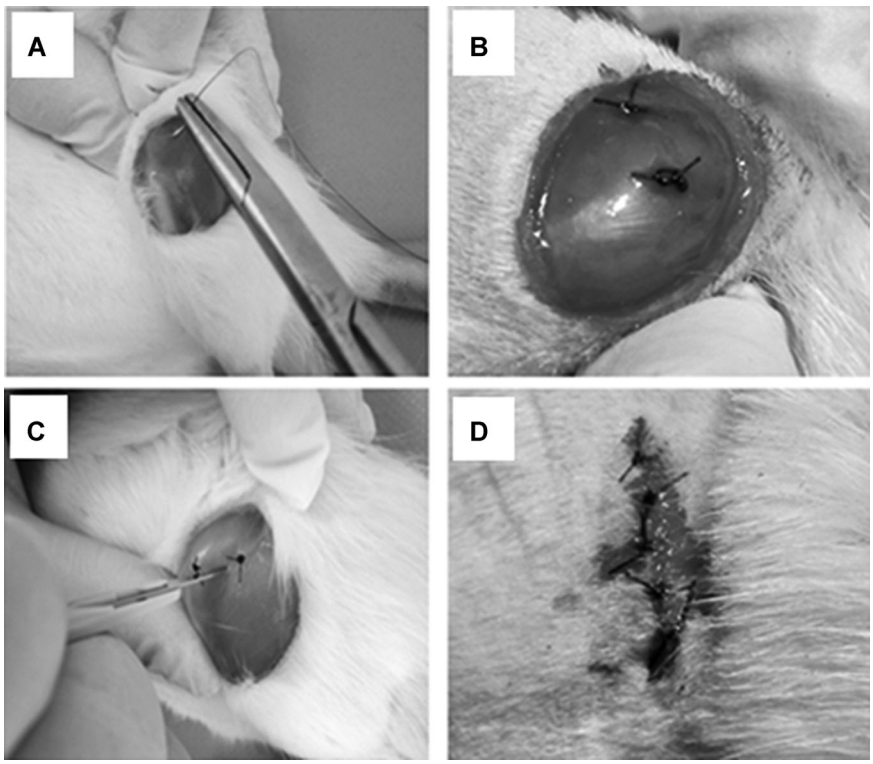

FIGURE 1 Operation procedure: A, rotation of the femur; B, premarking for the lesion with filament; C, placement of the lesion; D, closure of the skin

2.4 | Histological analyzes

The marked muscle area was extracted as described and fixed in paraformaldehyde. For histological analyses, the fixed tissues were embedded in paraffin blocks (Roti-Plast Paraffin; Carl Roth GmbH) and cut into 5-μm slices (Mikrotom 2030; Reichert-Jung, Heidelberg, Germany). The sections were then either stained with hematoxylin and eosin (H&E) or immunohistochemistry was performed using specific antibodies against CD31. The centralized nuclei were marked and counted by two independent researchers.

2.5 | Immunohistochemistry

The sections were first treated with Proteinase K (Dako S3020; Agilent Technologies), then blocked for 20 minutes with 3% H_2O_2 followed by a 30 minutes incubation step with horse serum (Biochrom GmbH, Berlin, Germany). CD31 mouse-monoclonal antibody (NB100-64796; Novus Biologicals) was incubated overnight at 4°C. The sections were first labeled with a biotinylated linker, followed by streptavidin-conjugated horseradish peroxidase, and finally counterstained with hematoxylin.

2.6 | Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics version 23. Data were considered as nonnormally distributed and univariate analysis of variance (Kruskal-Wallis) was applied followed by the Mann-Whitney *U* test. For all analyses, a $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Gene expression analyses

The gene expression levels of *MyoD*, *NCAM*, *Myf5*, and *Myosin* in the treated muscle tissue were determined 2, 4, and 7 days after surgery (Figure 2). Gene expression of *MyoD* and *Myf5* was slightly upregulated in rats treated with diclofenac when compared with

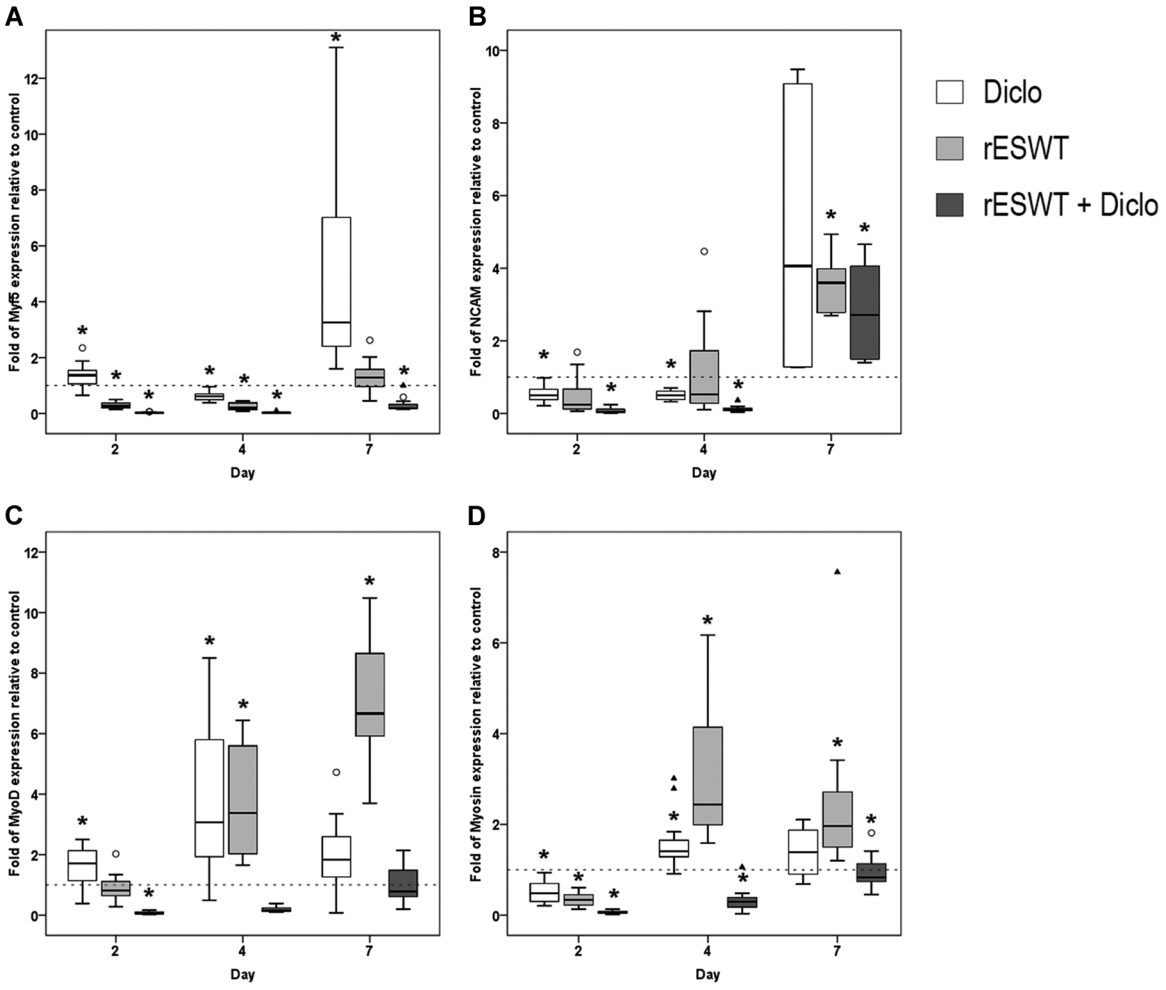

FIGURE 2 Gene expression of *Myf5* (A), *NCAM* (B), *MyoD* (C), and *Myosin* (D). Changes in *MyoD* and *Myosin* were detected on day 4 after monotherapies diclofenac or shockwave. Shockwave monotherapy resulted in an upregulation of *NCAM*, *MyoD*, and *Myosin*, even after 7 days. The combination of both single therapies resulted in a downregulation of *Myosin*, *MyoD*, *Myf5*, and *NCAM*. n = 9, $^*P < .05$. rESWT, radial extracorporeal shockwave application

the untreated control 2 days after surgery. All other groups demonstrated the downregulation of these two genes at the same time point. *NCAM* and *Myosin* gene expression decreased in all treatment groups (rESWT and diclofenac, both individually and combined) compared with control.

In contrast, the monotherapies, either diclofenac or rESWT, resulted in a significant upregulation of *MyoD* and *Myosin* expression after 4 days, and for rESWT alone after 7 days, whereas the combined therapy did not result in a positive effect on *MyoD* gene expression. *NCAM* and *Myf5* were only upregulated 7 days after surgery, and in the case of *Myf5* only after diclofenac treatment, whereas the expression of *NCAM* was enhanced after rESWT alone as well as with the combined therapy (Figure 2). The gene expression analysis of the satellite cell marker *Pax7* was also performed (data not shown) but no expression changes were detected in any of the treated groups, or at any of the time points when compared with the control. Only *NCAM* and *Myosin* expression were upregulated 7 days after surgery following the combined therapy.

3.2 | Histological analyses—H&E staining

H&E staining was performed for all groups on days 2, 4, and 7 after surgery. Differences could be observed between the treated groups and the untreated control on all days. Two days after surgery, few mononucleated cells (MNCs) could be observed in the lesion of the control group (untreated lesion). In contrast, around the lesions of the treated groups (diclofenac, rESWT, or combined therapy) large infiltrations of MNCs could be observed (Figure 3). The lighter stained regions show the degenerated fibers, which are more prominent in the untreated control group.

At 4 days following surgery, the number of MNCs in the control group increased when compared with the number of MNCs observed at

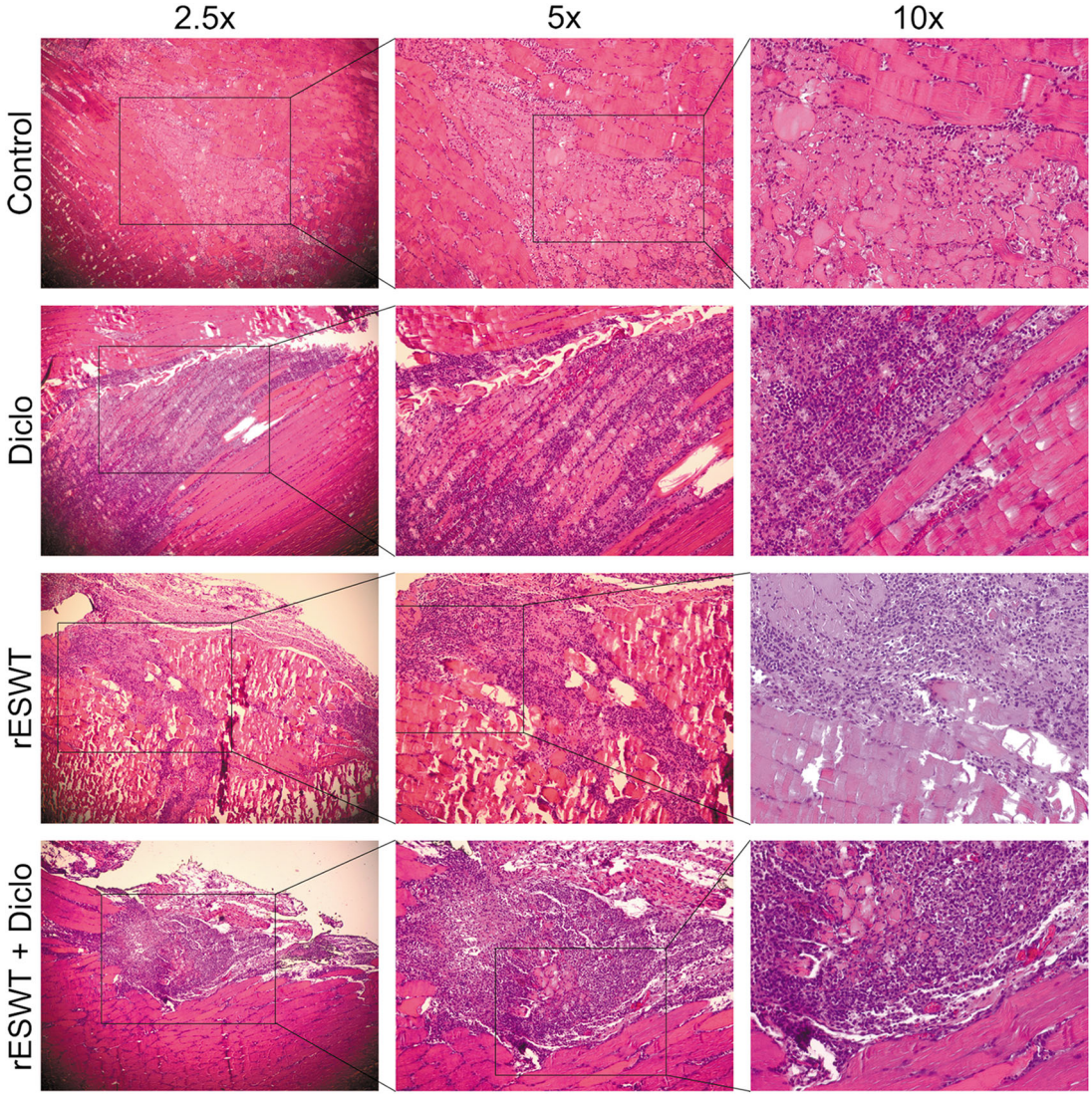

FIGURE 3 Hemotoxylin and eosin staining of muscle tissue 2 days after surgery. Mononucleated cells are visualized as dark purple cells. rESWT, radial extracorporeal shockwave application [Color figure can be viewed at wileyonlinelibrary.com]

2 days after surgery. However, in comparison with the treated groups, the number of MNCs was still lower. In addition, fatty deposits were observed within the untreated lesion. A direct comparison of the treated groups confirmed that there was a higher concentration of MNCs in the groups treated with rESWT alone, or with the combined therapy. The quantity of MNCs seen 2 days after surgery in the groups treated with diclofenac, rESWT, or both, is similar to the number of MNCs observed four days after surgery (Figure 4). Moreover, scar tissue formation could be observed, particularly in the control group, and to a lesser extent in the groups treated with diclofenac.

By day 7 after injury, the phenotype had altered. In the control group, a high number of MNCs could be detected, but within these cells, newly formed fibers were observed. The number of MNCs decreased significantly in all treated groups when compared with day 4 after surgery. This effect was most pronounced in the group treated with rESWT, where only a low number of MNCs could be detected. The number of MNCs in the group treated with diclofenac and the combined therapy was marginally higher when compared with the rESWT group but seemed to be lower than on day 4. Regenerating fibers could be detected in all therapy groups, indicating regeneration of the damaged muscle (Figure 5). The highest amount of regenerated muscle tissue was observed in the group treated with rESWT alone. This group also showed the highest number of centralized nuclei (36), which are an indicator of regenerating muscle fibers. The lowest number of centralized nuclei was detected in the group that received the combined therapy (13). In the diclofenac group, approximately 17 centralized nuclei could be counted.

Interestingly, fatty infiltration of the damaged tissue was neither observed after treatment with rESWT, nor after the combined therapy, when compared with the control and the diclofenac group.

The qualitative observations were verified quantitatively (Figure 6) by measuring the area occupied by the MNCs.

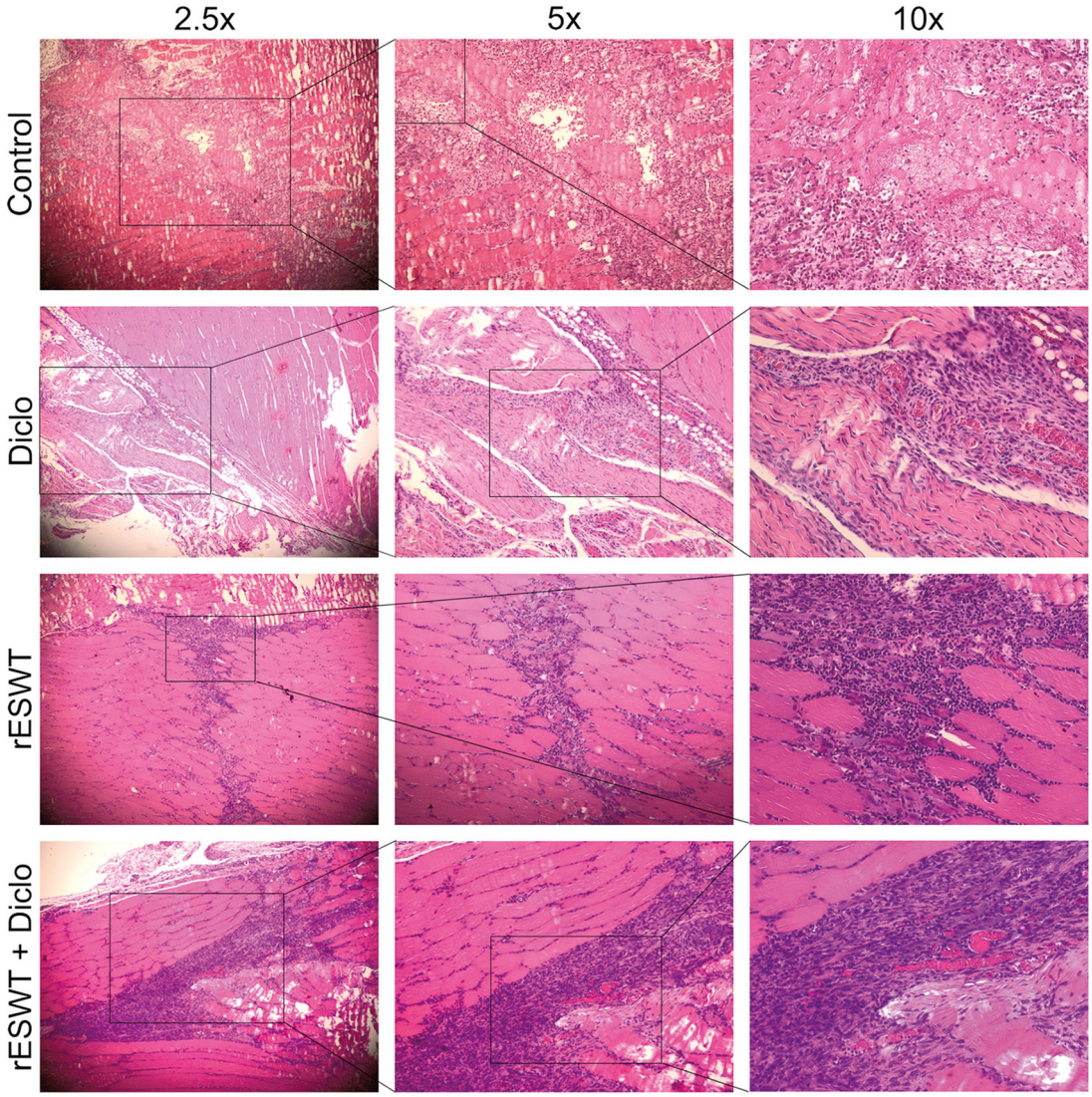

FIGURE 4 Hemotoxylin and eosin staining of muscle tissue four days after surgery. Mononucleated cells are visualized as dark purple cells. Mononucleated cells are found in all groups, but fewer are found in the treatment groups when compared with the control. Scar tissue can be detected in the control group and to a lower extent in the groups treated with diclofenac. rESWT, radial extracorporeal shockwave application [Color figure can be viewed at wileyonlinelibrary.com]

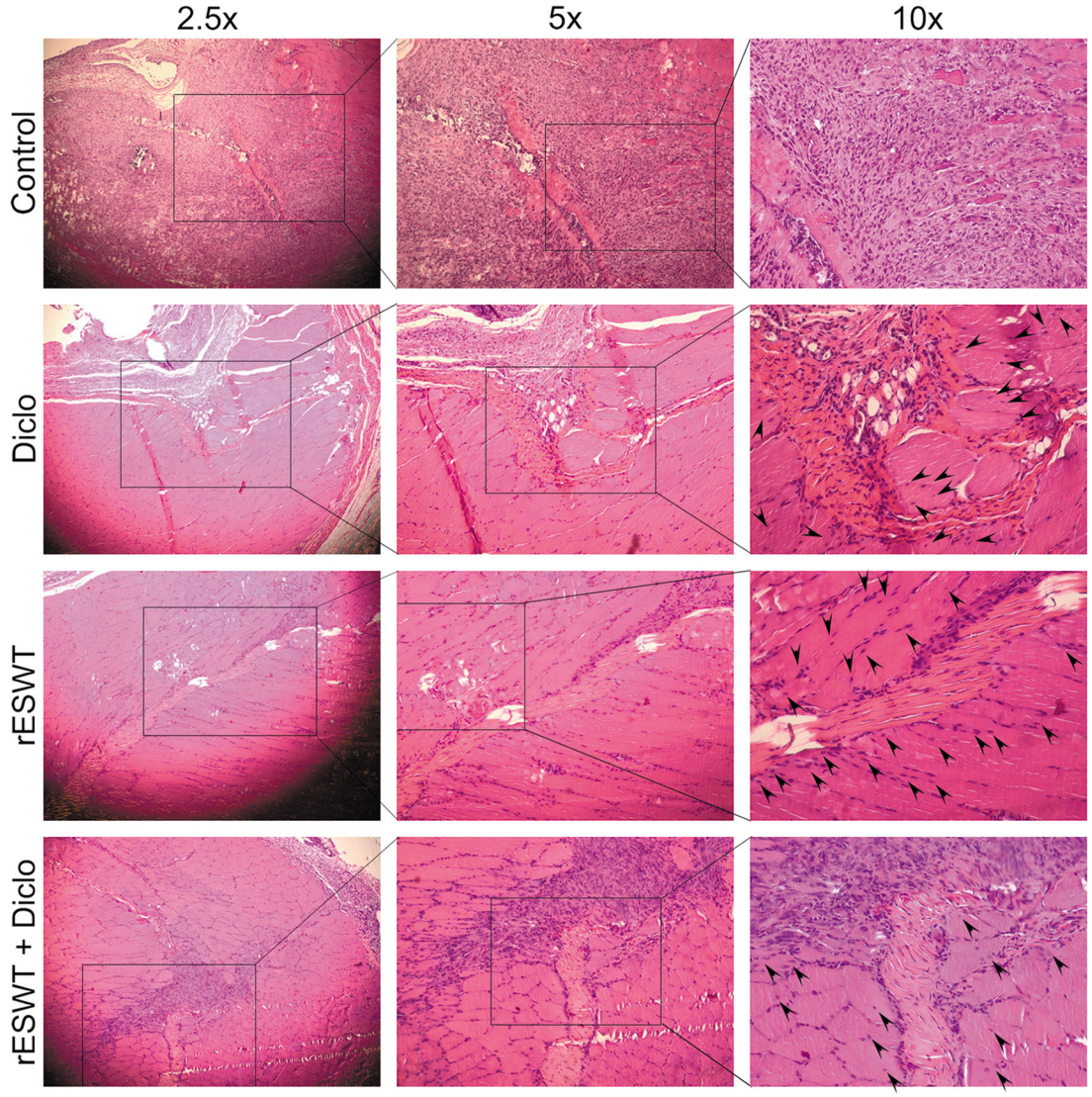

FIGURE 5 Hemotoxylin and eosin staining of muscle tissue 7 days after surgery. On day 7, the number of mononucleated cells was smaller in all treated groups when compared with the control. Regenerating tissue and new fibers can be observed in all treated groups, when compared with the untreated group, presenting as an accumulation of cells around the injury. Centralized nuclei are marked by arrows. rESWT, radial extracorporeal shockwave application [Color figure can be viewed at wileyonlinelibrary.com]

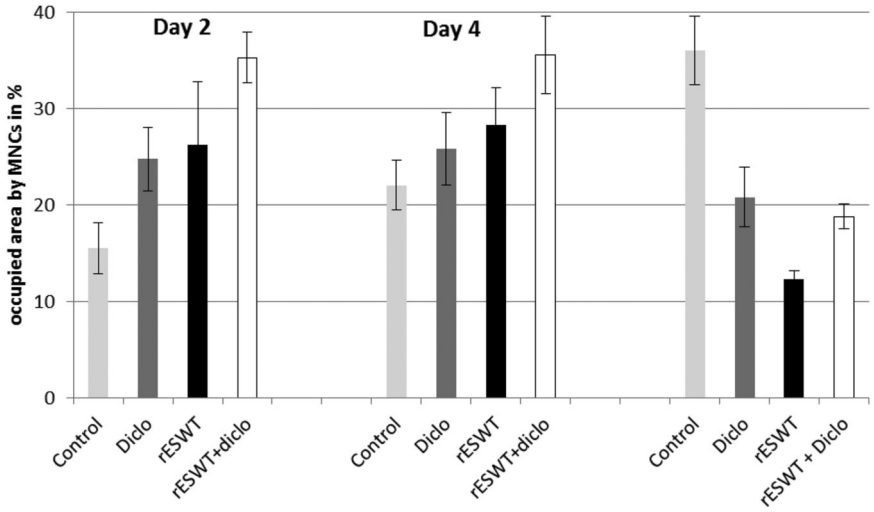

FIGURE 6 Quantitative analyses measuring the percentage area occupied by mononucleated cells, employing ImageJ software. rESWT, radial extracorporeal shockwave application

3.3 | CD31 staining

To demonstrate whether rESWT induced angiogenesis after muscle injury, immunohistochemical staining for CD31 was performed for all groups at 2, 4, and 7 days after surgery. In all groups, CD31-positive cells could only be detected sporadically when compared with positive liver tissue control (Figure 7). No differences could be observed between the therapy groups and the control group (without treatment), indicating that rESWT had no effect on angiogenesis in regenerating muscle.

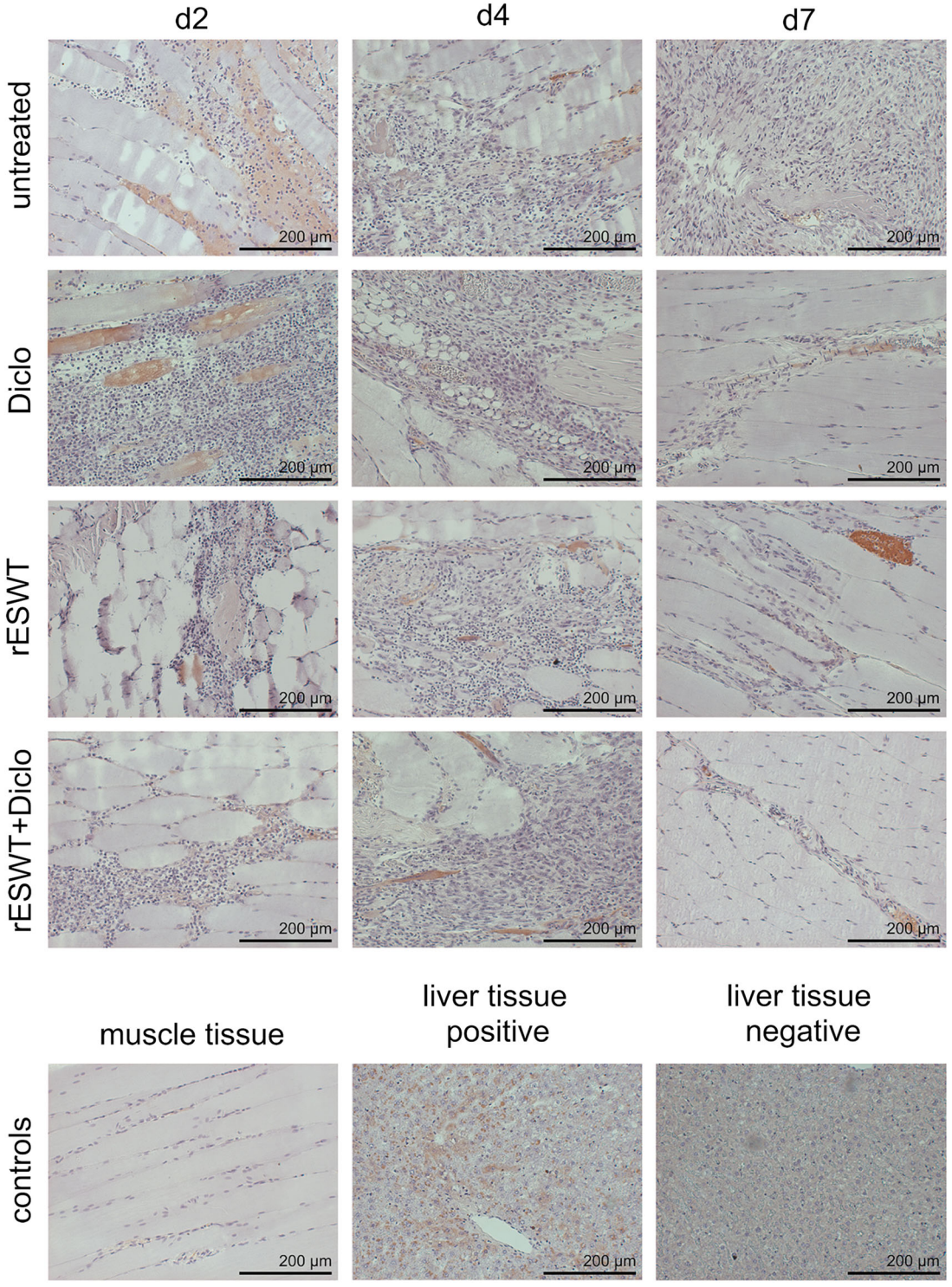

FIGURE 7 CD31 staining (brown cells) on days 2, 4, and 7 after surgery. CD31-positive cells could only be detected sporadically in all groups when compared with positive liver tissue control. rESWT, radial extracorporeal shockwave application [Color figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

Shockwave therapy is a noninvasive therapy applied for musculoskeletal disorders. However, its efficiency is only partly proven and in most cases, no proof of concept exists. The same applies to treatment with diclofenac. Although diclofenac is a standard therapy used after muscle injury, hardly any studies exist that analyze the effect of diclofenac on muscle cells or muscle injury, either in vitro or in vivo. To our knowledge, this is the first study to compare the effects of radial shockwave application and diclofenac treatment alone, or in combination, in a surgical rat model of muscle regeneration after injury.

We used a surgically induced muscle injury in our rat model, which is comparable to muscle injuries experienced by professional athletes or football players. Other options for inducing muscle injuries include injection with cardiotoxin, a neurotoxin, which depolarizes and destroys muscle cells or muscle contusion models.[19,21] In our surgical model we employed a scalpel to create muscle lesions of 3 mm in depth, which is comparable with a standard muscle injury in athletes.

4.1 | Gene expression

Satellite cells are able to differentiate into myoblasts and then further to myotubes, which merge and form new muscle fibers and fascicles.[26] Muscle regeneration is characterized by the expression of muscle-specific transcription factors, for example, *Pax7*, *Myf5*, and *MyoD*. During differentiation of the satellite cells into myoblasts, *Pax7* is downregulated, whereas *Myf5* and *MyoD* remain constantly expressed in myoblasts[27] and enhance the cellular self-regeneration.[28] In our study, we found a low, not regulated expression of *Pax7* in all treated groups and at all time points, when compared with the control group. Only one other published study has analyzed *Pax7* expression in a mouse model following shockwave application.[29] Although a significant increase in *Pax7* gene expression was noted by this group in the early phase of regeneration, after low-intensity shockwave treatment (Li-SWT), they did not observe any differences in the number of *Pax7* positive cells when compared with the untreated control. As they employed cardiotoxin to induce muscle lesion and used low-intensity shockwave therapy, their results may not be directly comparable with our current study. In addition, muscle healing is accelerated in rats when compared with mice,[11] and by 2 days after surgery the early phase has already ended. Only two studies exist that analyze the effect of NSAIDs on satellite cells and these studies have reported the opposite effects. Mackey et al[30] demonstrated activation of satellite cells following the ingestion of NSAID in young men, whereas Mikkelsen et al[31] observed an inhibiting effect on satellite cell proliferation in human skeletal muscle after exercise. As these studies were performed in humans, and species-specific differences could exist,[18] these results are also difficult to compare with our data.

After the fusion of myoblasts to myotubes, myogenin and myosin heavy chain are expressed and are markers for regenerating muscle.[32] NCAM is another specific marker expressed in myoblasts, myotubes, and muscle fibers during muscle regeneration.[33,34] In our present study, diclofenac and rESWT significantly increased the myogenic factors in the late phase of regeneration (after 4 days in rats). *NCAM* expression was only significantly increased on day 7, either after rESWT treatment or after the combined therapy. One other study has analyzed *NCAM* expression after the administration of NSAIDs. Mackey et al[35] found no changes in *NCAM* expression levels 8 days after administration in humans. However, in our study, we found an effect 7 days after administration in rats. Considering that a week in a rat's life is equivalent to at least 20 times that of the time period in humans,[36] 8 days post-NSAID administration may be too early to detect any expression changes in human subjects.

MyoD was significantly upregulated at both 2 and 4 days after surgery by diclofenac treatment, and at 4 and 7 days after rESWT application, indicating regenerating muscle. In contrast, *MyoD* expression was significantly downregulated on day 2, when compared with the untreated control, following treatment with the combination of both monotherapies. *Myf5* was significantly increased at both 2 and 7 days after treatment with diclofenac. An upregulation of *Myosin* was observed 4 and 7 days after rESWT application, but only on day 4 after diclofenac treatment (compared with untreated control), indicating that new muscle fibers were formed in regenerating muscle. In contrast, *Myosin* gene expression was significantly downregulated after the combined therapy on days 2 and 4 but significantly upregulated after 7 days.

As the results on days 2 and 4 after muscle lesion generation do not follow a logical pattern, we have focused on the long-term outcome observed after 7 days. Seven days in a rat are equivalent to 7 months in humans,[36] and therefore this time point should be appropriate for studying long-term outcomes. At 7 days after incision, with the exception of *Myf5*, shockwave therapy demonstrated the best effect, followed by monotherapy diclofenac, with the least effect being observed after the combined therapy. Similar changes in gene expression have been observed after rESWT treatment of primary human myoblasts in vitro.[17] It was surprising that the combined therapy showed the least effect on muscle regeneration. However, diclofenac acts through the inhibition of the COX-2 pathway and inhibition of inflammatory pathways, rather than directly on muscle regeneration. In fact some studies have demonstrated that NSAIDs have a negative effect on muscle protein synthesis and myogenic cell regeneration. Shockwaves decrease COX-2 expression even further, as shown in a cystitis model[37] and in macrophages.[38] This could explain the negative effect of the combined therapy when compared with the monotherapies.

The significant increase in *MyoD* expression observed on day 4 could indicate the beginning of the regeneration process and the onset for the differentiation of myoblasts to myotubes, supported by diclofenac and rESWT (Figure 2C). *Myf5* significantly decreased after 4 days (Figure 2A) and *MyoD* increased at the same time point,

suggesting that a differentiation process was underway to form new fibers. Our results are in contrast to those of Hansen et al,[29] who demonstrated that *MyoD* expression was not altered in mice after Li-SWT treatment, indicating that the Li-SWT treatment could not induce muscle differentiation. The suppression of *MyoD* expression observed after the combined therapy could indicate that the healing process is repressed and that damaged tissue could not regenerate due to the double repression induced by a combination of the two therapies. Nevertheless, these data are not really comparable to our study.

4.2 | Histological staining

Histological staining confirmed the overall results that mono- or combined therapies of diclofenac or rESWT induced muscle regeneration to a higher extent when compared with the untreated groups. Especially, evident at 7 days after surgery, and after treatment with rESWT, the injured areas are permeated by regenerating fibers. In the diclofenac and combined therapy groups, mononuclear cells are detected in higher amounts when compared with the rESWT group but are still much less than in the untreated group. These results are comparable with the study by Zissler et al.[39] who used cardiotoxin to generate muscle lesions in rats and treated them with extracorporeal shockwaves (ESWTs). They also performed histological studies and found that ESWT stimulated regeneration of skeletal muscle tissue. This is comparable with the effects of rESWT on the surgical muscle lesions employed in our study. The difference between ESWT and rESWT is the penetration depth into the tissue, physical characteristics, and the technique of how the impulses are generated.[40,41]

We also observed the regeneration processes histologically at 7 days after surgery by the formation of centralized nuclei, indicating regenerated fibers and the fusion of myoblasts to newly formed muscle fibers around the lesion. As shown in Figure 5 no centralized nuclei were detected in the control group compared with diclofenac (~17), rESWT (~36), and the combined therapy (~13).

4.3 | Angiogenesis

Previous studies in the literature suggest that shockwaves improve angiogenesis, vascularization, or microcirculation.[42,43] In a hindlimb ischemia rat model a mobilization of CD31/CD34-positive endothelial cells was demonstrated after rESWT, indicating an influence of this treatment on angiogenesis and vascularization.[42] Kisch et al[43] confirmed this by showing an improvement of muscular microcirculation after repetitive shockwave application. Both studies found high numbers of CD31 and CD34 cells in blood perfusion after SWT. Li-SWT of human myoblasts, and in a model of mouse skeletal muscle injury, also revealed significantly increased expression of angiogenic and myogenic genes.[29] However, Hansen et al[29] could not detect any changes in blood vessel density in mouse skeletal muscle after Li-SWT. In agreement with the study by Hansen, we did not observe more CD31-positive cells after rESWT treatment when compared with the nontreated control. Hence, we believe that rESWT directly affects muscle cells. This hypothesis is further supported by our in vitro study, where we could show that rESWT modulated viability and gene expression in primary human skeletal muscle cells.[17]

4.4 | Limitation of the study

The present data support the application of rESWT or diclofenac as monotherapies to treat muscle injuries but do not support the use of both treatments at the same time. Although these results are underpinned by a number of experimental approaches, the study has several potential limitations that need to be considered.

To reduce the animal numbers, we used control rats and muscle sections from another study that employed the same muscle model. The muscle tissues were excised on days 1, 3, and 7 instead of days 2, 4, and 7 in the treatment groups. Although we believe that the difference of 1 day between the control and treated tissues would not have much influence on the interpretation of results, it is worth mentioning.

As the aim of our study was to analyze muscle regeneration, we primarily focused on gene expression changes of myogenic transcription factors. Including an analysis of fibrotic, fatty, and inflammatory genes, such as transcription factors from the COX pathway and/or angiogenic factors, could have given further information and is part of a follow-up study.

Another limitation is the therapy schedule. We attempted to translate the treatment schedule used for professional soccer teams to our in vivo model. However, there are many differences between the healing and regeneration processes present in rats vs humans, suggesting that additional schedules employing different frequencies, time points, and concentrations should be investigated.

Finally, this study did not investigate if there were any changes in protein expression as a result of the alterations in gene expression, and these data might strengthen our results. The protein expression analyses are a part of the follow-up study as well as further immunohistochemical staining, for example, collagen.

5 | CONCLUSION

Our study shows that muscle regeneration is supported by rESWT and to a lower extent by diclofenac applied in mono- or combined therapies in a controlled in vivo study imitating muscle lesions in sports. rESWT alone demonstrated the best effect on regenerating fibers in injured muscle tissue. Therefore, we recommend prospective clinical studies to analyze the effect of rESWT after sports trauma to

induce muscle regeneration and to shorten the rehabilitation time for sports professionals after muscle injury.

ACKNOWLEDGMENTS

The authors would like to acknowledge the substantial contribution of Angelika Ackermann and her invaluable assistance with the histology. They would also like to thank Electro Medical Systems for kindly providing a Swiss DolorClast device for their experiments; Lisa Nauth for providing the negative control; Dr Cherie Stayner, University of Otago, Dunedin, New Zealand, for scientific English editing of this manuscript. This study was supported by the level I funding of the University Medical Center of the Johannes Gutenberg-University of Mainz.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

SM and UR conceived and planned the experiments; EL and AK carried out the experiments; EL and UR wrote the manuscript in consultation with PR, PD, and SM. All authors have read and approved the final submitted manuscript.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ORCID

Ulrike Ritz http://orcid.org/0000-0001-8936-3227

REFERENCES

1. Feeley BT, Kennelly S, Barnes RP, et al. Epidemiology of National Football League training camp injuries from 1998 to 2007. *Am J Sports Med*. 2008;36:1597-1603.
2. Hallén A, Ekstrand J. Return to play following muscle injuries in professional footballers. *J Sports Sci*. 2014;32:1229-1236.
3. Delos D, Maak TG, Rodeo SA. Muscle injuries in athletes: enhancing recovery through scientific understanding and novel therapies. *Sports Health*. 2013;5:346-352.
4. Stevens KJ, Crain JM, Akizuki KH, Beaulieu CF. Imaging and ultrasound-guided steroid injection of internal oblique muscle strains in baseball pitchers. *Am J Sports Med*. 2010;38:581-585.
5. Hamid MSA, Yusof A, Mohamed Ali MR. Platelet-rich plasma (PRP) for acute muscle injury: a systematic review. *PLoS One*. 2014;9:e90538.
6. Terada S, Ota S, Kobayashi M, et al. Use of an antifibrotic agent improves the effect of platelet-rich plasma on muscle healing after injury. *J Bone Joint Surg Am*. 2013;95:980-988.
7. Ekstrand J, Hägglund M, Waldén M. Epidemiology of muscle injuries in professional football (soccer). *Am J Sports Med*. 2011;39:1226-1232.
8. Ota S, Uehara K, Nozaki M, et al. Intramuscular transplantation of muscle-derived stem cells accelerates skeletal muscle healing after contusion injury via enhancement of angiogenesis. *Am J Sports Med*. 2011;39:1912-1922.
9. Karalaki M, Fili S, Philippou A, Koutsilieris M. Muscle regeneration: cellular and molecular events. *In Vivo*. 2009;23:779-796.
10. Järvinen TAH, Järvinen TLN, Kääriäinen M, Kalimo H, Järvinen M. Muscle injuries: biology and treatment. *Am J Sports Med*. 2005;33:745-764.
11. Borrione P, Grasso L, Chierto E, et al. Experimental model for the study of the effects of platelet-rich plasma on the early phases of muscle healing. *Blood Transfus*. 2014;12(suppl 1):s221-s228.
12. Ioppolo F, Rompe JD, Furia JP, Cacchio A. Clinical application of shock wave therapy (SWT) in musculoskeletal disorders. *Eur J Phys Rehabil Med*. 2014;50:217-230.
13. Malay DS, Pressman MM, Assili A, et al. Extracorporeal shockwave therapy versus placebo for the treatment of chronic proximal plantar fasciitis: results of a randomized, placebo-controlled, double-blinded, multicenter intervention trial. *J Foot Ankle Surg*. 2006;45:196-210.
14. Schmitz C, DePace R. Pain relief by extracorporeal shockwave therapy: an update on the current understanding. *Urol Res*. 2009;37:231-234.
15. Hofmann A, Ritz U, Hessmann MH, Alini M, Rommens PM, Rompe JD. Extracorporeal shock wave-mediated changes in proliferation, differentiation, and gene expression of human osteoblasts. *J Trauma*. 2008;65:1402-1410.
16. Hofmann A, Ritz U, Rompe J-D, Tresch A, Rommens PM. The effect of shock wave therapy on gene expression in human osteoblasts isolated from hypertrophic fracture non-unions. *Shock Waves*. 2015;25:91-102.
17. Mattyasovszky SG, Langendorf EK, Ritz U, et al. Exposure to radial extracorporeal shock waves modulates viability and gene expression of human skeletal muscle cells: a controlled in vitro study. *J Orthop Surg*. 2018;13:75.
18. Morelli KM, Brown LB, Warren GL. Effect of NSAIDs on recovery from acute skeletal muscle injury: a systematic review and meta-analysis. *Am J Sports Med*. 2018;46:224-233.
19. de Almeida P, Tomazoni SS, Frigo L, et al. What is the best treatment to decrease pro-inflammatory cytokine release in acute skeletal muscle injury induced by trauma in rats: low-level laser therapy, diclofenac, or cryotherapy? *Lasers Med Sci*. 2014;29:653-658.
20. Wolfson TS, Hamula MJ, Jazrawi LM. Impact of diabetes mellitus on surgical outcomes in sports medicine. *Phys Sportsmed*. 2013;41:64-77.
21. Dos Santos LS, Saltorato JC, Monte MG, et al. PBMT and topical diclofenac as single and combined treatment on skeletal muscle injury in diabetic rats: effects on biochemical and functional aspects. *Lasers Med Sci*. 2018.
22. Contreras-Muñoz P, Fernández-Martín A, Torrella R, et al. A new surgical model of skeletal muscle injuries in rats reproduces human sports lesions. *Int J Sports Med*. 2016;37:183-190.
23. Hardy D, Besnard A, Latil M, et al. Comparative study of injury models for studying muscle regeneration in mice. *PLoS One*. 2016;11:e0147198.
24. Tomazoni SS, Frigo L, Dos Reis Ferreira TC, et al. Effects of photobiomodulation therapy and topical non-steroidal anti-inflammatory drug on skeletal muscle injury induced by contusion in rats-part 2: biochemical aspects. *Lasers Med Sci*. 2017;32:1879-1887.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402-408.

26. Hirata A, Masuda S, Tamura T, et al. Expression profiling of cytokines and related genes in regenerating skeletal muscle after cardiotoxin injection. *Am J Pathol*. 2003;163:203-215.
27. Sachidanandan C, Sambasivan R, Dhawan J. Tristetraprolin and LPS-inducible CXC chemokine are rapidly induced in presumptive satellite cells in response to skeletal muscle injury. *J Cell Sci*. 2002;115:2701-2712.
28. Kablar B, Rudnicki MA. Skeletal muscle development in the mouse embryo. *Histol Histopathol*. 2000;15:649-656.
29. Hansen LK, Schrøder HD, Lund L, Rajagopal K, Maduri V, Sellathurai J. The effect of low intensity shockwave treatment (Li-SWT) on human myoblasts and mouse skeletal muscle. *BMC Musculoskelet Disord*. 2017;18:557.
30. Mackey AL, Rasmussen LK, Kadi F, et al. Activation of satellite cells and the regeneration of human skeletal muscle are expedited by ingestion of nonsteroidal anti-inflammatory medication. *FASEB J*. 2016;30:2266-2281.
31. Mikkelsen UR, Langberg H, Helmark IC, et al. Local NSAID infusion inhibits satellite cell proliferation in human skeletal muscle after eccentric exercise. *J Appl Physiol*. 2009;107:1600-1611.
32. Pavlath GK, Dominov JA, Kegley KM, Miller JB. Regeneration of transgenic skeletal muscles with altered timing of expression of the basic helix-loop-helix muscle regulatory factor MRF4. *Am J Pathol*. 2003;162:1685-1691.
33. Péault B, Rudnicki M, Torrente Y, et al. Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. *Mol Ther*. 2007;15:867-877.
34. Illa I, Leon-Monzon M, Dalakas MC. Regenerating and denervated human muscle fibers and satellite cells express neural cell adhesion molecule recognized by monoclonal antibodies to natural killer cells. *Ann Neurol*. 1992;31:46-52.
35. Mackey AL, Kjaer M, Dandanell S, et al. The influence of anti-inflammatory medication on exercise-induced myogenic precursor cell responses in humans. *J Appl Physiol*. 2007;103:425-431.
36. Sengupta P. The laboratory rat: relating its age with human's. *Int J Prev Med*. 2013;4:624-630.
37. Wang H-J, Lee W-C, Tyagi P, Huang CC, Chuang YC. Effects of low energy shock wave therapy on inflammatory moleculars, bladder pain, and bladder function in a rat cystitis model. *Neurourol Urodyn*. 2017;36:1440-1447.
38. Sukubo NG, Tibalt E, Respizzi S, Locati M, d'Agostino MC. Effect of shock waves on macrophages: a possible role in tissue regeneration and remodeling. *Int J Surg*. 2015;24:124-130.
39. Zissler A, Steinbacher P, Zimmermann R, et al. Extracorporal shock wave therapy accelerates regeneration after acute skeletal muscle injury. *Am J Sports Med*. 2017;45:676-684.
40. Schmitz C, Császár NBM, Milz S, et al. Efficacy and safety of extracorporeal shock wave therapy for orthopedic conditions: a systematic review on studies listed in the PEDro database. *Br Med Bull*. 2015;116:115-138.
41. Schmitz C, Császár NB, Rompe J-D, Chaves H, Furia JP. Treatment of chronic plantar fasciopathy with extracorporeal shock waves (review). *J Orthop Surg*. 2013;8:31.
42. Tepeköylü C, Wang F-S, Kozaryn R, et al. Shock wave treatment induces angiogenesis and mobilizes endogenous CD31/CD34-positive endothelial cells in a hindlimb ischemia model: implications for angiogenesis and vasculogenesis. *J Thorac Cardiovasc Surg*. 2013;146:971-978.
43. Kisch T, Wuerfel W, Forstmeier V, et al. Repetitive shock wave therapy improves muscular microcirculation. *J Surg Res*. 2016;201:440-445.

How to cite this article: Langendorf EK, Klein A, Drees P, Rommens PM, Mattyasovszky SG, Ritz U. Exposure to radial extracorporeal shockwaves induces muscle regeneration after muscle injury in a surgical rat model. *J Orthop Res*. 2020;38:1386–1397. https://doi.org/10.1002/jor.24564