

Original Article

Effect of extracorporeal shock wave on proliferation and differentiation of equine adipose tissue-derived mesenchymal stem cells in vitro

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Abstract: Mesenchymal stem cells are regarded as common cellular precursors of the musculoskeletal tissue and are responsible for tissue regeneration in the course of musculoskeletal disorders. In equine veterinary medicine extracorporeal shock wave therapy (ESWT) is used to optimize healing processes of bone, tendon and cartilage. Nevertheless, little is known about the effects of the shock waves on cells and tissues. Thus, the aim of this study was to investigate the influence of focused ESWT on the viability, proliferation, and differentiation capacity of adipose tissue-derived mesenchymal stem cells (ASCs) and to explore its effects on gap junctional communication and the activation of signalling cascades associated with cell proliferation and differentiation. ASCs were treated with different pulses of focused ESWT. Treated cells showed increased proliferation and expression of Cx43, as detected by means of qRT-PCR, histological staining, immunocytochemistry and western blot. At the same time, cells responded to ESWT by significant activation (phosphorylation) of Erk1/2, detected in western blots. No significant effects on the differentiation potential of the ASCs were evident. Taken together, the present results show significant effects of shock waves on stem cells in vitro.

Keywords: Horse, shock wave, stem cell, proliferation, differentiation

Introduction

Disorders of the musculoskeletal system, such as osteoarthritis, tendinopathies, and fractures, are the main reasons for the early retirement and euthanasia of horses [1, 2]. A conventional treatment option for these disorders consists of a combination of stall rest, physiotherapy, hoof care, and long-term pain management using nonsteroidal anti-inflammatory drugs. Other, more recent treatment options include implantation of cells and tissue-engineered constructs in bone, cartilage and tendon [3, 4]. Mesenchymal stem cells are ideal cells for implantation. Equine ASCs possess excellent therapeutic potential for tissue regeneration, have self-regenerative abilities [5], and have the potential to differentiate into several musculoskeletal-related cell lineages [6-8]. In recent years, several clinical investigations

have revealed promising results in the stem cell treatment of a variety of orthopedic disorders in horses [9-13].

An alternative treatment for musculoskeletal disorders is the application of ESWT. The shock wave is an acoustic wave that is characterized by an extremely high amplitude and short rise time and is followed by a longer, low-magnitude negative wave [14]. Originally, ESWT was used for lithotripsy of uroliths and gall stones in humans and animals [15-18]. Since the early 1990s, ESWT has been commonly applied in the treatment of musculoskeletal disorders, such as tendinopathies and osteoarthritis, in equine medicine [19-26].

The precise therapeutic and biological effects of ESWT on tissues or cells, however, are not yet completely understood. It has been sug-

gested that ESWT induces cell growth and enzymatic activities [19], leads to an induction of neovascularisation [27] has an osteostimulatory effect [28], and has a direct effect on membrane permeability and viability of chondrocytes and on the structure of cartilage [29]. It is assumed that ESWT could possibly lead to an activation of endogenous stem cells.

Implantation of stem cells in combination with shock wave therapy could be considered as a new, alternative therapy for orthopaedic diseases. In order to evaluate this possibility, we investigate in the work presented here whether various doses of ESWT might affect the morphological and biochemical properties of mesenchymal stem cells by analysing its effects on proliferation, apoptosis, and differentiation of equine ASCs.

Material and methods

The chemical reagents were obtained from PAA (PAA, Germany) or Sigma (Sigma, Germany) unless otherwise noted.

Tissue harvest and isolation of ASCs

Subcutaneous adipose tissue was collected from the region localized above the dorsal gluteal muscles of three mixed-breed horses (aged, mean \pm SD, 7.8 \pm 2.3 years) as previously described [7]. The samples were obtained from horses being slaughtered at the local abattoir. All samples were collected within 20 min post mortem. ASCs were isolated by collagenase type I (Biochrom AG, Germany) digestion as previously described [7].

ESWT

ASCs from passages 1 to 4 were used for ESWT. Since shock wave conditions have a strong effect on the behaviour and the intercellular contacts of the cells [30], adherent cells were treated after reaching 80% confluence. Cells with an optimal morphology (spindle-shaped) and vitality were used for the experiments. Hypertrophic cells were excluded from the experiment, because an increased cell size can be taken as evidence of senescence. The ASCs with visible intracellular granules were excluded as well, because this can be a sign for dedifferentiation. The proliferation capacities and the differentiation potential were randomly tested.

The control group did not receive any extracorporeal shock wave treatment, while Experimental Group 1 (EG 1000/9) received 9 pulses of 1000 shock waves and Experimental Group 2 (EG 2000/3) received 3 pulses of 2000 shock waves, according to standard means of delivery in horse patients [31, 32].

Cell morphology

ASCs were examined using a phase contrast microscope and the Axiovision image analysis system (Carl Zeiss, Germany).

MTT assay

Cell viability was evaluated using a colorimetric MTT assay measuring reduction power (AppliChem, Germany). The cells (15×10^3 cell/well) were cultured for 24 h and 48 h at 37°C with 5% CO₂. Thereafter, 0.5 mg/ml of MTT reagent was added to the Dulbecco's Modified Eagle's Medium (DMEM with 1000 mg/L Glukose) and the cells were incubated for 4 h at 37°C with 5% CO₂. DMSO (200 μ l) was then added to dissolve the water-insoluble formazan salt. Quantification was performed with a spectrophotometer at 570 nm (ELISA reader; Tecan, Germany).

Senescence assessment (β -galactosidase assay)

Processes of cell aging can be indicated by assessing β -galactosidase (β -Gal) activity. For the determination of changes caused by senescence, cells (15×10^3 cell/well) were harvested immediately after shock wave application and incubated for 24 h at 37°C with 5% CO₂. Cells were then washed in 0.1 M phosphate-buffered saline (PBS) and subsequently lysed in 0.1% Triton in PBS. After centrifugation at 4°C and 13.000 g for 15 min, samples were incubated with β -Gal solution for 16 h at 37°C. The absorbance of the dye at 570 nm was measured with a spectrophotometer.

Immunofluorescence staining

The immunofluorescence staining was performed as previously described [8]. To visualize the actin cytoskeleton, cells were incubated with Alexa Fluor 594 phalloidin (6.6 μ M final concentration). Cell nuclei were counterstained using the Hoechst nuclear stain H33334. Cell proliferation was assessed by the detection of

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Ki67 antigen. For the detection of apoptotic cells, cells were stained with propidium iodide (1.5 mM final concentration).

The method of detection of connexin 43 (Cx43) was similar to that for the detection of Ki67 antigen [8]. The cells were incubated with the primary antibody (mouse anti-connexin43, diluted 1:100 by vol.; DAKO, Germany) overnight at 4°C and were then exposed to the secondary antibody (goat anti-mouse, diluted 1:200 by vol.; DAKO, Germany) for 30 min. Fluorescence images of ASCs were obtained using the Axiovision image analysis system (Carl Zeiss, Germany).

Preparation of cell lysates

Cells were lysed using a commercially available cell lysis buffer according to the manufacturer's protocol (Cell Signaling Technology, Germany). All lysis steps were carried out on ice. After centrifugation at 4°C and 13,000 for 15 min, protein content in the supernatant was determined using the bicinchoninic acid (BCA) protein assay reagent kit (Pierce, IL, USA).

SDS-PAGE and immunodetection of Cx43 and total and activated Erk1/2

A total of 10 µg of proteins from cell lysates were separated by SDS-PAGE using slab gels containing 10% acrylamide and 0.3% N, N'-methylene-bis-acrylamide. Biotinylated molecular weight markers (Cell Signaling Technology, Germany) were run parallel. After electrophoresis, the proteins were electro-blotted at 500 mA for 30 min onto nitrocellulose membranes (Schleicher & Schuell, Germany). After blotting, the membranes were incubated with the appropriate primary antibody in a dilution of 1:500 (phosphorylated (p-Erk1/2) or total Erk1/2 (t-Erk1/2) or anti-Cx43 (mouse monoclonal anti-Cx43, Invitrogen) overnight at 4°C. The visualization of the ERK1/2 proteins was performed using the appropriate horseradish peroxidase-conjugated IgG (1:2000 in TBS-T containing 5% nonfat milk) provided by the enhanced chemiluminescence (ECL) kit (Amersham-Pharmacia, Germany). The membranes incubated with the C43 antibody were further incubated with a biotinylated secondary antibody (goat-anti-mouse IgG, in a dilution of 1:1000, DAKO Hamburg, Germany). Then the peroxidase conjugated avidine (ABC)-complex

(Vector, Burlingame, CA, USA) was applied. A horseradish peroxidase-conjugated anti-biotin IgG (Cell Signaling Technology, Germany) to detect the biotinylated molecular weight markers was included in the incubation medium at a dilution of 1:2000. After film exposure, the density of the resultant bands was analyzed using a digital documentation system (Adobe Photoshop CS5, Ireland). As a negative control, the primary antibody was replaced by non-immune serum.

In vitro differentiation

Adipogenic and osteogenic differentiation was performed in a monolayer as described previously [8]. Cells were plated at a density of 3×10^3 cells/cm². All cells were incubated in a humidified atmosphere at 37°C with 5% CO₂ for various periods. The medium was changed three times a week.

Adipogenic differentiation

ASCs were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 1% antibiotics (penicillin 100 U/ml, streptomycin 0.1 mg/ml; P/S), 1 µM dexamethasone, 10 µM insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 100 µM indomethacin. Adipogenic differentiation was confirmed on day 14 using an Oil Red O stain as an indicator of intracellular lipid accumulation and adipocyte-specific gene expression.

Osteogenic differentiation

The cells used for osteogenic differentiation were cultured in osteogenic medium. DMEM was supplemented with 10% FCS, 1% P/S, 0.05 mM ascorbic acid-2-phosphate, 10 mM β-glycerophosphate and 0.1 µM dexamethasone for 3 weeks. Osteogenesis was demonstrated by accumulation of mineralized calcium phosphate (calcified extracellular matrix, ECM) assessed by von Kossa stain.

Chondrogenic differentiation

Chondrogenic differentiation was performed in a 3D culture with a cell density of 3×10^5 cells/pellet. For determination of chondrogenesis, the cells were centrifuged in 15 ml Falcon tubes at 200 g for 5 min. Cells were incubated overnight at 37°C, 5% CO₂ in a humidified atmosphere. Formed cell pellets were cultivated in chondrogenic differentiation medium supple-

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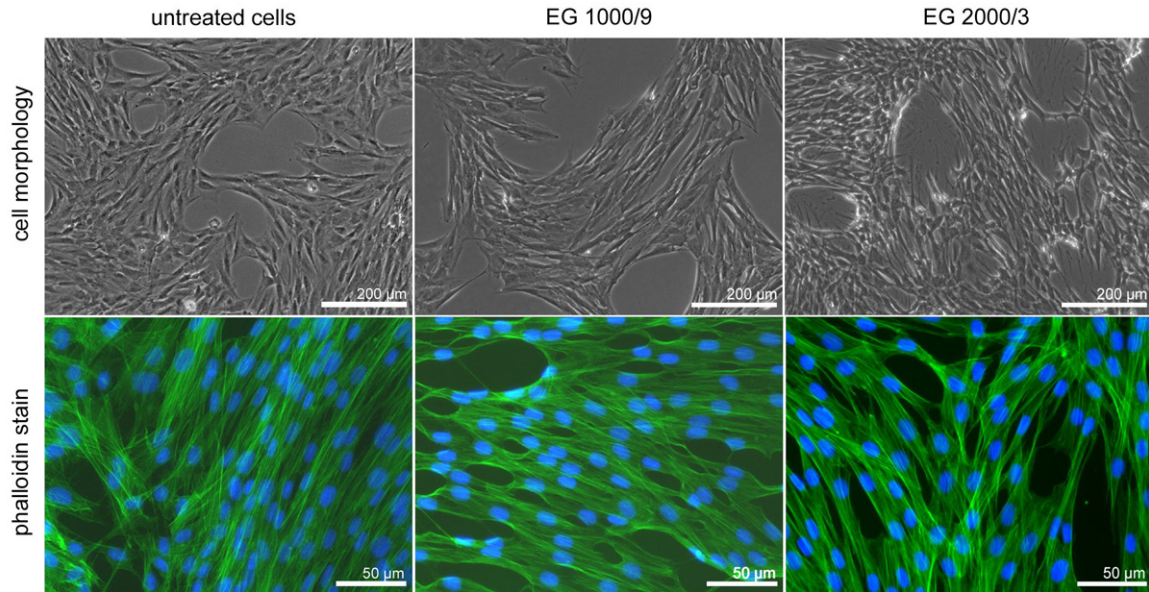


Figure 1. Cell morphology of ASCs. Phase-contrast microscopy of untreated ASCs revealed highly spindle-shaped, fibroblastic cell morphology. The cells of EG 1000/9 and EG 2000/3 were less spindle-shaped and the cell extensions were shortened. The cytoskeleton of the ASCs was stained with phalloidin.

mented with DMEM, 1% FCS, 1% P/S, 0.05% ITSx100, 50 μM ascorbic acid, 100 nM dexamethasone and 10 ng/ml TGF β 1 for 3 weeks. After culture, pellets were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm thin sections. The sections were deparaffinised with xylene and ethanol. To detect proteoglycan synthesis as an indicator of cartilage production, sections were stained with Alcian blue. Counterstaining was performed with nuclear fast red.

Quantitative realtime-RT-PCR (qRT-PCR)

Total RNA was extracted using TRI[®] Reagent according to the manufacturer's protocol (Sigma, Germany). The RNA concentration was adjusted to 200 ng/ μl , treated with a recombinant DNase I (Roche, Germany), and subsequently reverse-transcribed using GeneAmp[®] Gold RNA PCR Core Kit according to the manufacturer's protocol (Applied Biosystems, Germany). The PCR primers and annealing conditions are listed in Table 1. Real-time RT-PCR was carried out on a CFX96 Realtime Cycler (Bio-Rad, Germany) using IQ SybrGreen Supermix (Bio-Rad) according to the following protocol: after an initial 3 min at 90°C amplification was accomplished by 40 cycles of heating for 15 sec at 90°C followed by 1 min 60°C, with a subsequent melting curve. Expression of

gene of interest was normalized to GAPDH expression. Data were analyzed using the CFX Manager software 1.6 (Bio-Rad) applying the $\Delta\Delta\text{CT}$ -method for relative gene expression.

Statistical analysis of data

One-way ANOVA was performed for comparison of groups. Subsequently, the groups were compared pairwise with the control by the Mann-Whitney U test followed by Dunnett's test. For all tests the statistical software program, SPSS19.0, was used (IBM, Germany).

Results

Cell morphology

The ASCs showed elongated, spindle-shaped cell bodies with 2-3 long, slim processes (**Figure 1**). Only a minor percentage of the shock wave-treated cells changed their morphology (approximately 10% within EG 1000/9 and approximately 20% within EG 2000/9). The cell processes shortened and the cell bodies gained volume, which may have been caused by a swelling process (**Figure 1**).

The visualization of the f-actin cytoskeleton was accomplished by phalloidin staining, where the alignment of directed and undirected fibers was assessed. A slightly increased alignment of

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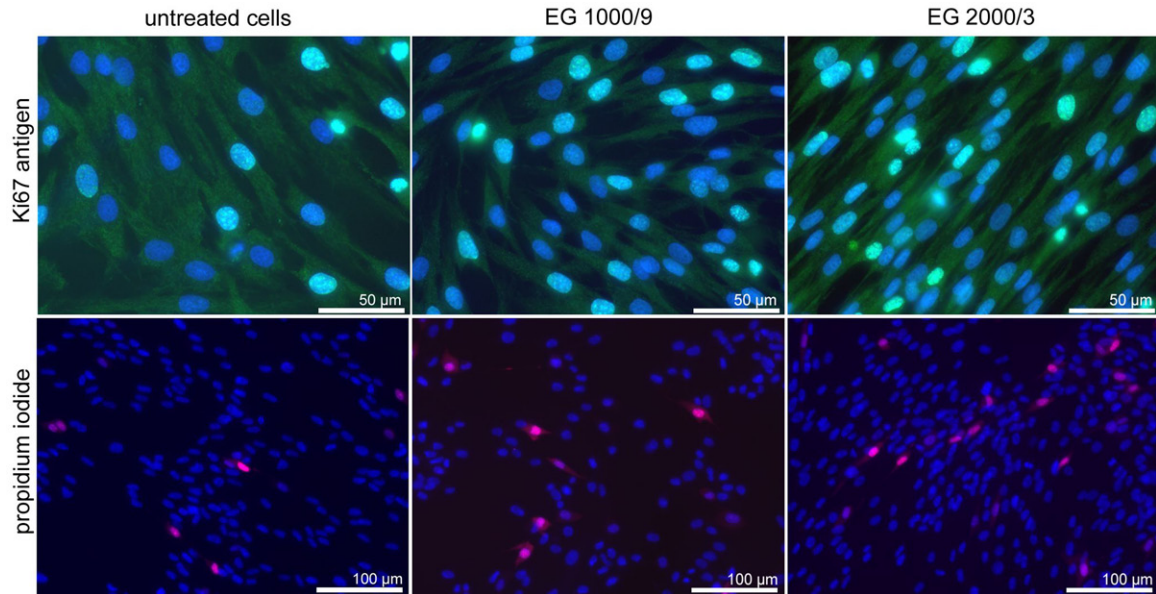


Figure 2. Cell proliferation and apoptosis. Cell proliferation was assessed by immunofluorescence with the Ki67 antibody. Upper panels: Turquoise-stained nuclei indicate proliferation. Lower panels: apoptosis was stained with propidium iodide; pink stained nuclei indicate apoptosis.

the f-actin fibers in the shock wave-treated cells was observed. However, no significant alterations could be detected (**Figure 1**).

Cell proliferation and viability

There was a significantly increased proliferation of the stem cells, which was determined utilizing the proliferation marker Ki67. Thus, the proliferation in cells of the control group it was about $25.01 \pm 2.5\%$, in the cells of EG 1000/9 at $33.08 \pm 2.4\%$ and in EG 2000/3 at $40.95 \pm 4.3\%$ (**Figures 2, 3A**). The results of the proliferation were confirmed by the MTT assay, so that a significant increase of the number of shock wave-treated cells compared with the untreated cells was clearly recognizable. Thus, in the cells of the control group, the absorbance after 24 h was about 0.45 ± 0.06 and after 48 h 0.98 ± 0.14 ; in the cells of EG 1000/9 after 24 h 0.73 ± 0.16 and after 48 h 1.28 ± 0.24 ; in EG 2000/3 after 24 h 0.84 ± 0.25 and after 48 h 1.92 ± 0.61 (**Figure 3B**).

Cell senescence and apoptosis

The effect of mechanotransduction of the shock wave on cell apoptosis was also examined. The cells of EG 2000/3 showed a significantly higher amount of cell apoptosis ($12 \pm 4.7\%$) than the cells of EG 1000/9 ($7.72 \pm 2.2\%$)

or the untreated cells ($3.34 \pm 0.28\%$) (**Figures 2, 3C**).

Cell senescence can be assessed by measuring β -Gal activity. Following, the density of the dye was measured. Relating to the untreated cells, the results of the β -Gal assay revealed no changes for the shock wave application at any time. Thus, the senescence in cells of the control group was 0.19 ± 0.01 , in the cells of EG 1000/9 at 0.19 ± 0.03 and in EG 2000/3 at 0.2 ± 0.04 (**Figure 3D**).

In vitro differentiation

The analysis of the differentiation potential indicates to what extent stem cells can be directed towards the adipogenic, osteogenic, or chondrogenic lineage; this can be determined by applying specific media and factors. Shock wave-treated cells showed a slightly higher potential for differentiation than untreated cells. The adipogenic differentiation in the treated groups was more pronounced in terms of both numbers of differentiated cells and size of fat vacuoles. Fat vacuoles were noted within 3-4 days of shock wave treatment in treated cells and within 5 days in untreated cells (**Figure 4**). Following induction of osteogenic differentiation, we observed a clear change according to the morphology. The shock wave-treated cells

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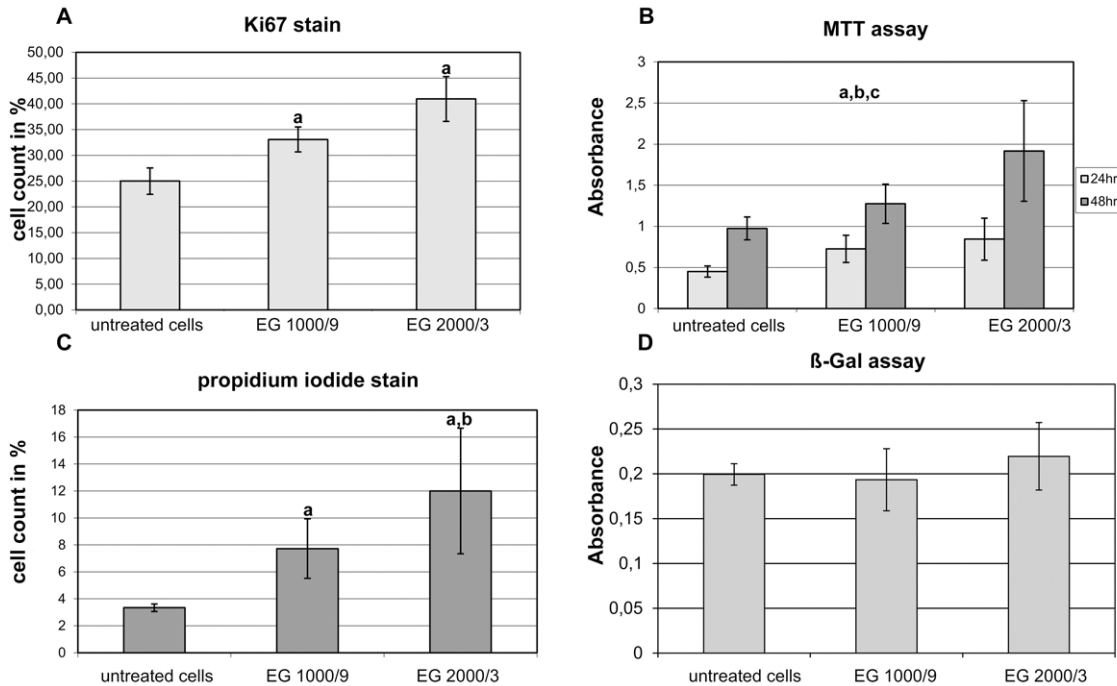


Figure 3. Quantitative analysis of cell proliferation, viability, apoptosis, and senescence. The treated cells showed a better proliferative capability as well as a higher apoptotic rate than the untreated cells. The data were compiled from triplicate determinations. The MTT assay showed a significantly higher cell proliferation of the treated cells than the untreated cells. The β -Gal assay showed that the cells of the three groups had nearly the same level of senescence. All values reflect the arithmetic mean \pm standard deviation; a: significantly higher than control, b: significantly higher than EG 1000/9, a, b, c: all samples are significant with each other.

of both treated groups acquired a polygonal shape within three days and cell aggregates and nodule formation were observed after 3-5 days of culture; in contrast, this was observed in the untreated cells after 5-7 days. Matrix mineralization and calcium accumulation were also more prominent in the treated groups (**Figure 4**). Within a day after seeding the ASCs in culture for chondrogenesis, three-dimensional aggregates were observed in all groups. Both treated and untreated cell pellets stained positive for alcian blue, indicating the presence of sulfated proteoglycans (**Figure 4**). No positive staining (for all three differentiation lineages) was observed in the cells cultured in growth medium (data not shown). However, the analysis of the adipo-, osteo- and chondrogenesis-relevant mRNA expression did not show any significant differences between the samples (**Figure 5**).

Effect of ESWT on Erk1/2

ESW treatment did not affect the expression of total Erk1/2 (**Figure 6**; t-Erk1/2). The expression of p-Erk1/2 (activated Erk1/2), however,

was significantly different between EG 2000/3 and EG 1000/9 and between treated cells of the both groups and the untreated cells (**Figure 6**). In EG 1000/9 cells, p-Erk1/2 was significantly higher than p-Erk1/2 in untreated or in EG 2000/3 cells.

Cx43 expression

Cx43 was detected by immunofluorescence staining in the untreated and both treated groups (**Figure 7A**). This can be confirmed by Western blot experiments (**Figure 7C**) showing a marginally higher expression of Cx43 in both treated groups (48.76% and 49.29%) compared to the control group (46.01%). However, looking at Cx43 mRNA expression a significant increase in Cx43 expression was observed in EG 1000/9 in comparison with the untreated cells and cells treated by EG 2000/3 (**Figure 7B**).

Discussion

ASCs have a high therapeutic potential and are therefore used in various areas of regenerative

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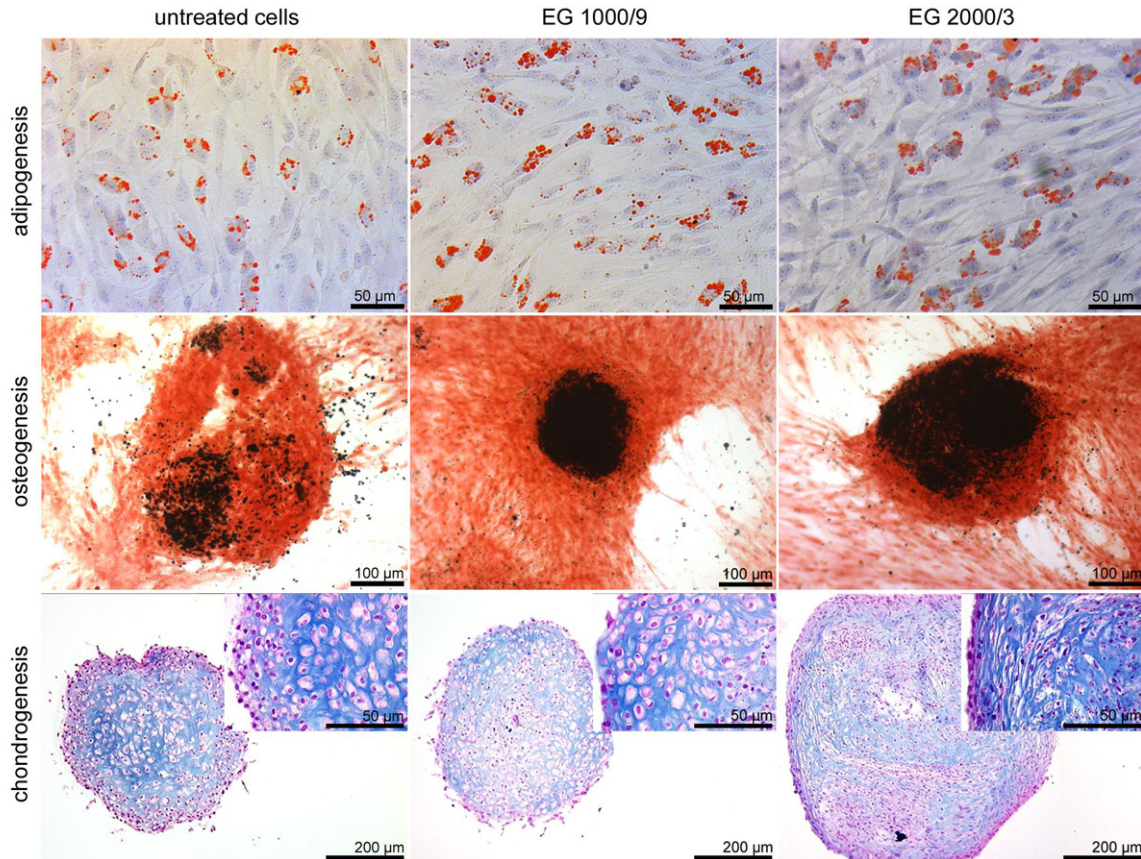


Figure 4. Cell differentiation. The adipogenic differentiation was detected by the formation of lipid droplets in the cytoplasm of cells stained with Oil Red O. Osteogenic differentiation was demonstrated by calcium deposition stained with von Kossa stain. Chondrogenic differentiation of ASCs was determined by a transformation from the fibroblastic to a chondrocyte-like appearance; formation of GAG-rich matrix and numerous chondrone-like areas were observed. The center of the pellet from EG 2000/3 became increasingly subjected to necrosis.

medicine. However, the success of this therapy is limited by the small number of stem cells that reach the target area. Therefore, the cells have to be conditioned for their *in vivo* application or to be modified for the transplantation niche. Recently, various forms of shock wave therapy have been applied in addition to a stem cell therapy. The mechanisms involved in shock wave therapy, however, are not yet completely understood and are often the subject of controversy. Thus, the aim of the present study was to examine whether behaviour and characteristics of equine ASCs can be modified under the influence of shock wave therapy. For that purpose the effects of ESWT on the cytoskeleton of ASC and on their proliferation and differentiation potential were analyzed. The results obtained show a distinct mechano-sensitive reaction of the equine ASCs to the application of shock wave treatment. Significantly different effects were observed between the treated

groups and the untreated group, which were consistent with results reported by other researchers [33-35]. Additionally, there are numerous reports about the effect of ESWT on different cells (chondrocytes, marrow stromal cells, tumor cells etc) [36-40]. Similar to our findings the authors of these papers report about an increase in the proliferation rate as well as about a dose- and impulse dependent cytotoxic effect. However, the increased proliferation rate especially of stem cells observed after shock wave application, as investigated in our study, suggests that the transplantation results might be influenced in a positive way if division- and reproduction speed of stem cells (proliferation rate) can be stimulated by ESWT *ex vivo* prior to transplantation.

It is well known that the cytoskeleton plays an active role in the process of apoptosis [41]. Thus, loosening the cells from their focal adhe-

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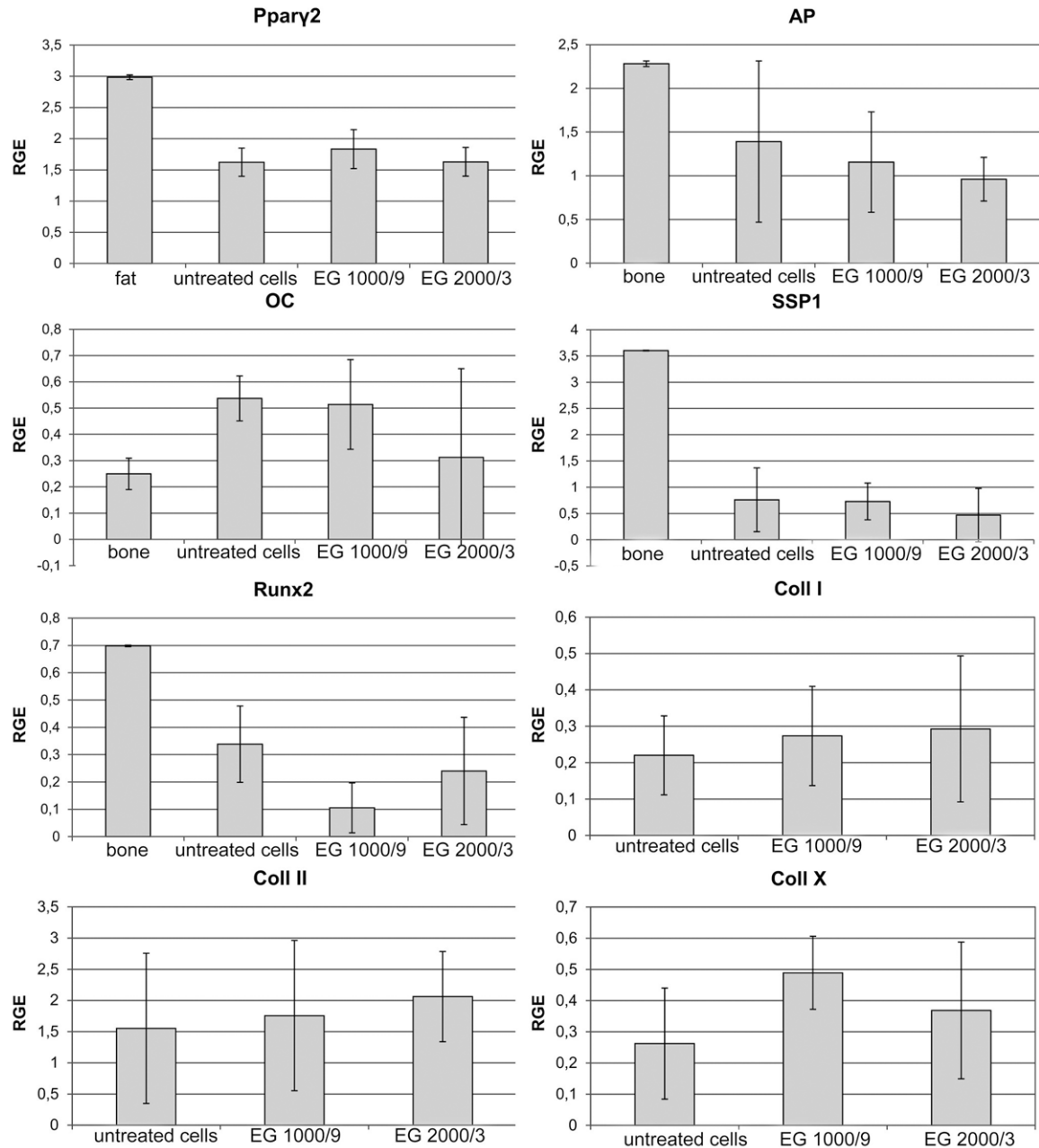


Figure 5. qRT-PCR after differentiation. mRNA expression of adipogenic (Pparγ2), osteogenic (AP, OC, SSP1, Runx2), and chondrogenic markers (Col I, Col III, and Col X). All values reflect the arithmetic mean \pm standard deviation. RGE: relative gene expression.

sion complexes as well as reorganization of the f-actin fibers and inhibition of actin dynamics following cell contraction are some of the first signs of apoptosis [18]. Our investigation showed significant effects of the application of shock wave treatment on cell apoptosis with increasing severity of the shock wave conditions. Furthermore, an alignment of the f-actin fibers was demonstrated by phalloidin staining

after shock wave application. Therefore, a correlation between the dynamics of the f-actin fibre formation and apoptosis can be assumed that is in accordance with the observations of other research groups [18]. Furthermore, different structures of the ASCs are altered during shock wave application, and these findings confirm that the use of the wrong shock wave conditions can result in cell death.

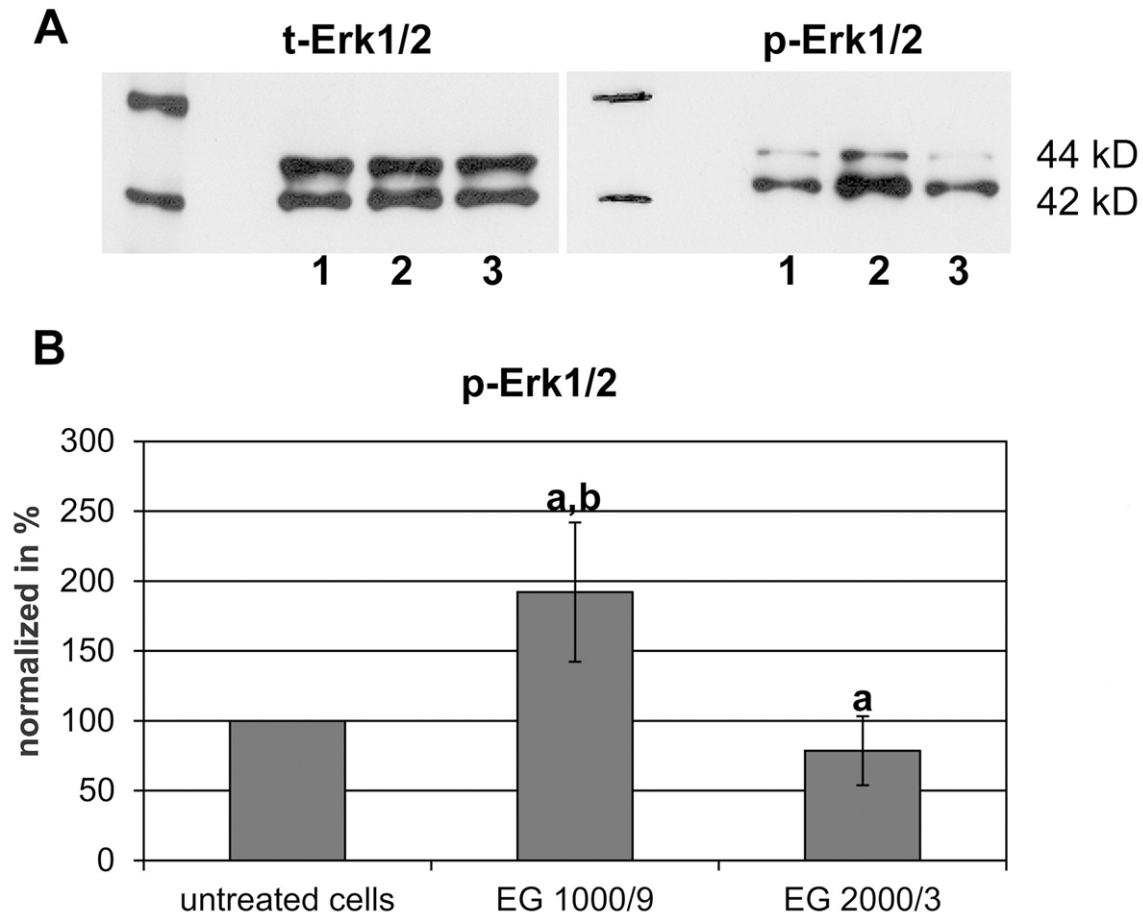


Figure 6. Erk1/2 expression. A: Western blot analysis of total- and phosphorylated-Erk1/2 expression in untreated (1), EG 1000/9 (2), and EG 2000/3 (3) groups. The p-Erk1/2 expression is significantly higher in the EG 1000/9 than in the control or EG 2000/3 group (n=6; mean \pm SEM). The value for the EG 2000/3 group is significantly smaller than that of the control group (n=6; mean \pm SEM); a: significantly higher than control, b: significantly higher than EG 2000/3.

Aging processes are reflected in the morphology of cells as well as in the activity of β -galactosidase [42]; therefore, we measured β -Gal activity to assess effects of the shock wave treatment on cellular aging. There were no differences in β -Gal activity between control and treated cells, thus allowing to assume that the treatment did not cause any senescence on ASCs.

A better differentiation potential of the cells after shock wave treatment was demonstrated for the adipogenic, osteogenic, and chondrogenic lineages (Figure 4) without any detectable changes in the mRNA expression of corresponding markers (Figure 5). The shock waves possibly stimulate the translation of the marker mRNAs into protein without any direct effect on mRNA synthesis. These results are strength-

ened by the fact that shock wave treatment caused an almost 2-fold stimulation of Erk1/2 in the EG 1000/9 group when compared with the untreated group. This effect was not observed in the EG 2000/3 group, however, demonstrating once again the importance of the correct choice of treatment. Since p-Erk1/2 is involved in the regulation of cell growth and differentiation, it is justified to assume that the observed activation of the kinase might be of significance for the mitogenic and differentiation responses of the ASCs. Activation of Erk1/2 in connection with differentiation and proliferation of mesenchymal stem cells has been described previously [43, 44].

The analysis of the cell-cell contacts was performed by examining the presence of Cx43, the most common Cx in mammals [45]. Cx43 has a

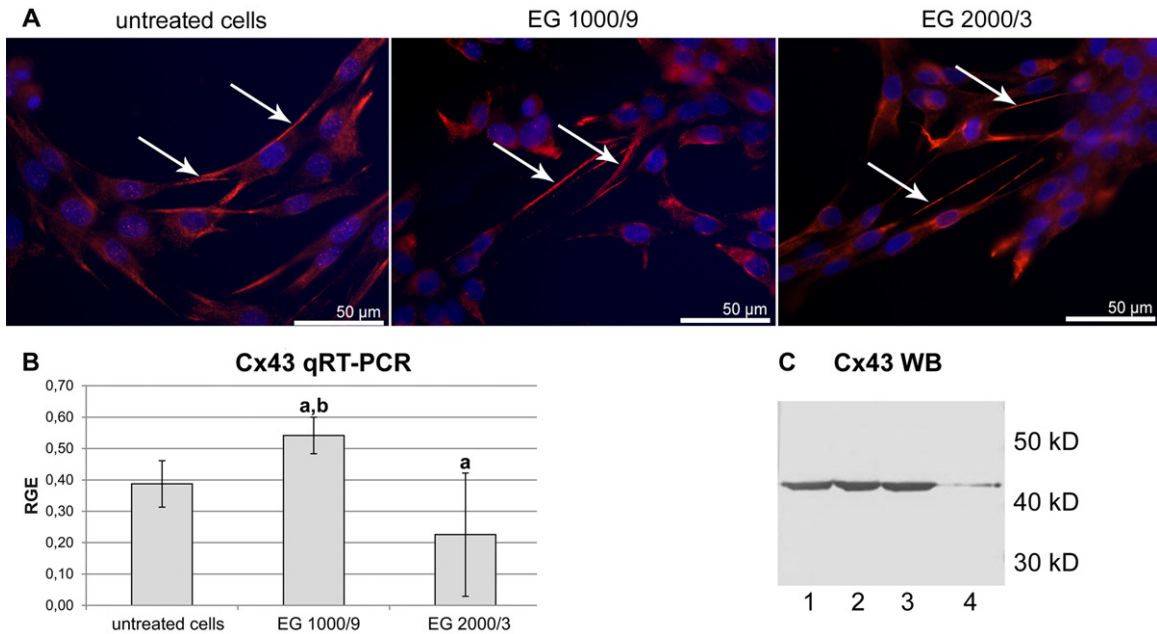


Figure 7. mRNA expression, immunofluorescence staining and Western blot analysis of Cx43. Cx43 protein expression was detected immunocytochemically in all three groups (arrows) (A). The Cx43 mRNA expression is significantly higher in the EG 1000/9 than in the control or EG 2000/3 group. All values reflect the arithmetic mean \pm standard deviation. RGE: relative gene expression; a: significantly higher than control, b: significantly higher than EG 2000/3 (B). Western blot analysis reveals an expression of Cx43 in both experimental groups as well as in the control group with a slightly higher expression in the ESWG treated groups (C). Lanes 1-3 as in A, lane 4 = equine heart as positive control.

role in the regulation of cell differentiation and cell proliferation [46, 47]. On the protein level, Cx43 was detected in the cells of untreated as well as slightly increased in both shock wave-treated groups, with a slightly higher expression in the EG 2000/3 group. Cx43-specific mRNA expression, however, was higher in the EG 1000/9 group than in EG 2000/3 or in untreated cells. These findings actually indicate that shock wave treatment has the potency to alter the expression of cell-cell contact proteins as shown here for the gap junction proteins.

Furthermore, these findings also confirm the results for proliferation, and differentiation, and reveal new aspects for fundamental research, which might be translated to clinical applications. Since the multiplication and cultivation of ASCs *ex vivo* allow for the production of a high number of stem cells that can be subjected to shock waves under ideal conditions without affecting other cells in the surrounding tissue, *ex vivo* pre-conditioning of equine stem cells by shock wave application followed by the re-implantation of these cells into tissue lesions might help to improve the treatment of orthopaedic disorders.

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