

RESEARCH ARTICLE

Extracorporeal shock wave therapy *in situ* – novel approach to obtain an activated fat graft

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Abstract

One of the mainstays of facial rejuvenation strategies is volume restoration, which can be achieved by autologous fat grafting. In our novel approach, we treated the adipose tissue harvest site with extracorporeal shock wave therapy (ESWT) in order to improve the quality of the regenerative cells *in situ*. The latter was demonstrated by characterizing the cells of the stromal vascular fraction (SVF) in the harvested liposuction material regarding cell yield, adenosine triphosphate (ATP) content, proliferative capacity, surface marker profile, differentiation potential and secretory protein profile. Although the SVF cell yield was only slightly enhanced, viability and ATP concentration of freshly isolated cells as well as proliferation doublings after 3 weeks in culture were significantly increased in the ESWT compared with the untreated group. Likewise, cells expressing mesenchymal and endothelial/pericytic markers were significantly elevated concomitant with an improved differentiation capacity towards the adipogenic lineage and enhancement in specific angiogenic proteins. Hence, *in situ* ESWT might be applied in the future to promote cell fitness, adipogenesis and angiogenesis within the fat graft for successful facial rejuvenation strategies with potential long-term graft survival.

KEYWORDS

adipogenesis, adipose tissue, angiogenesis, autologous fat grafting, extracorporeal shock wave therapy (ESWT), stromal vascular fraction (SVF)

1 | INTRODUCTION

The shape of the human face depends on many factors: the osseous facial skeleton, muscles, fat, connective tissue and skin. Nevertheless, adipose tissue together with the superficial fascia builds up the basic structure and contour of the human face. During facial aging, volume loss due to adipose and connective tissue atrophy occurs, followed by the sagging of structures and the formation of wrinkles (Amar & Fox, 2011; Sandhofer & Schauer, 2015). Hence, restoring volume by the injection of fillers is one of the mainstays of facial rejuvenation strategies. In contrast to traditional fillers, including hyaluronic acid and carboxymethylcellulose, which act only temporarily, autologous adipose tissue has the potential for permanent restoration combined with the additional advantage of improved quality of the overlying skin (Coleman & Katzel, 2015).

Autologous fat grafting was described for the first time in 1893 by Gustav A. Neuber (Neuber, 1893). Recently it has experienced a comeback in cosmetic and plastic surgery due to its relative ease of application associated with comparatively low risks (Gir, Oni, Brown, Mojallal, & Rohrich, 2012). The beneficial effect of autologous fat grafting could not only be shown for facial rejuvenation but also for body contour improvement and breast augmentation after mastectomies (Calabrese, Orzalesi, Casella, & Cataliotti, 2009; Gentile et al., 2012; Jiang, Li, Duan, Dong, & Wang, 2015; Luo et al., 2013). Although fat grafting has been constantly developed, certain drawbacks, such as fat resorption, limited survival of the graft followed by partial necrosis, fibrosis and calcification have to be faced in the clinics (Luo et al., 2013). Some of them might be attributed to improper harvesting and processing techniques and can be partially circumvented by adhering to the following principles: gentle harvesting of fat to preserve the adipose tissue structure, centrifugation to remove non-viable components and to concentrate the fat, and delivery of adipose tissue in small aliquots to facilitate adequate blood supply and thus maximize graft take and

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graft retention up to 65% (Coleman & Katznel, 2015). However, standard protocols regarding fat harvesting (cannula size, manual, machine-assisted), processing (aspiration, infiltration), purification and transplantation are still missing (Gir et al., 2012; Suszynski, Sieber, Van Beek, & Cunningham, 2015). Furthermore, the main factor influencing long-term graft survival is still a matter of debate. Whereas older studies concentrated on the viability of mature adipocytes in the graft, there has been a shift towards the interaction among the different components of fat grafts, including adipose stem and precursor cells, endothelial cells and their precursors, as well as the cells' secretome influencing graft survival. Whether these recently proposed laboratory parameters are really correlated with graft survival and, hence, volume retention in humans has still to be proven (Tuin et al., 2016).

In 2006, a novel approach, known as cell assisted lipotransfer, was implemented. Supplementing the fat graft with therapeutic cells derived from adipose tissue, such as the stromal vascular fraction (SVF), which includes the adipose-derived stromal/stem cells (ASC), can improve the survival rate of transplanted fat grafts (Yoshimura et al., 2008). It has been shown that cell assisted lipotransfer can reduce postoperative atrophy in breast augmentation (Salgarello, Visconti, & Farallo, 2010) and enhance angiogenesis of the grafts (Gentile et al., 2012; Jiang et al., 2015). The latter is an important feature as early and abundant vascularization is a prerequisite for nutrition supply and integration inside the surrounding tissue (Garza et al., 2014; Luo et al., 2013). As cell properties might change with isolation procedure, the comparison of the outcome of clinical trials may be difficult. In our novel approach, we hypothesize that application of extracorporeal shock wave therapy (ESWT) at the adipose tissue harvest site might precondition the cells inside the fat graft in such a way that the regenerative potential is enhanced.

ESWT was clinically implemented 30 years ago as an effective treatment to disintegrate urinary stones. In contrast to stone disintegration, ESWT for modulating regeneration is performed with lower energy (Tandan & Reddy, 2011). However, this technology has also emerged as an effective non-invasive treatment approach for several other indications, including musculoskeletal disorders such as tendinopathies and bone defects (delayed/non-union of bone fractures, avascular necrosis of the femoral head) (Saggini et al., 2008), problematic soft tissue wounds (Antonic, Mittermayr, Schaden, & Stojadinovic, 2011) or erectile dysfunction (Lei et al., 2013). Potential regenerative mechanisms exerted as biological responses to therapeutic shock waves include initialization of neovascularization, recruitment of mesenchymal stem cells, stimulating cell proliferation and differentiation, anti-inflammatory and anti-microbial effects, as well as suppression of nociception (Mittermayr et al., 2012).

Also, in cosmetic and aesthetic medicine, ESWT has been discovered for the treatment of patients with cellulite, lipoedema and lymphoedema (Cebicci et al., 2016; Sandhofer, 2015; Siems, Grune, Voss, & Brenke, 2005). In cellulite and lipoedema patients, ESWT significantly improved the biomechanical skin properties, leading to smoothing of the dermis and hypodermis surface (Siems et al., 2005). This effect is, among others, caused by remodelling of collagen in the ESWT-treated regions (Angehrn, Kuhn, & Voss, 2007).

Concerning cells, ESWT has been shown to positively affect cell viability and proliferation of various cell types, including mesenchymal

stem cells from adipose tissue and bone marrow, primary tendon cells and endothelial progenitors (de Girolamo et al., 2014; Peng et al., 2008; Raabe et al., 2013; Weihs et al., 2014; Zhao et al., 2013). This effect is triggered through immediate adenosine triphosphate (ATP) release that subsequently binds to purinergic receptors acting via downstream Erk1/2 signalling on cell proliferation (Chen et al., 2004; Weihs et al., 2014). Apart from increased growth potential, enhanced differentiation from progenitors into mature cell types has also been observed (Chen et al., 2004; Leone et al., 2016; Muzio et al., 2014; Schuh et al., 2016; Zhai et al., 2016). By contrast, Raabe and co-workers (Raabe et al., 2013) did not find an effect of ESWT on the differentiation potential of equine ASC. The positive effect of ESWT on cells might also be attributed to increased expression and secretion of growth factors playing a role in regeneration settings (de Girolamo et al., 2014; Muzio et al., 2014; Peng et al., 2015; Zhao et al., 2013). On top of that, ESWT has not only been demonstrated to improve the quality of cells, but also to enhance cell yield when the nerve tissue was treated with ESWT before isolating cells, which might also be crucial for realizing cellular therapies (Schuh et al., 2016).

For this study, seven female patients undergoing routine liposuction were treated with ESWT before the actual procedure. Subsequently, from the liposuction material, the SVF was isolated and analysed regarding cell yield, ATP content, proliferative capacity, surface marker profile, differentiation potential and secretory protein profile. Within this study, SVF and derived cultured cells were used as an indicator for the functionality of future fat grafting approaches for facial rejuvenation using ESWT on the harvesting site. We hypothesized that *in situ* ESWT might positively affect the cell yield and the cell quality.

2 | MATERIALS AND METHODS

2.1 | Radial ESWT *in situ*

Seven female donors were treated locally below the iliac crest 1 week and 1 h before liposuction with radial ESWT (Z WavePro, Zimmer, Neu-Ulm, Germany) using two doses of 2000 pulses with 120 mJ/cm² and 16 Hz (Figure 1). The donors were treated on their left side with ESWT and remained untreated on their right side (control). The use of human adipose tissue was approved by the local ethical board with patient's consent. Subcutaneous adipose tissue was obtained during routine outpatient liposuction procedures under local tumescence anaesthesia. Afterwards, isolation of SVF from the liposuction waste was continued with enzymatic digestion. Briefly, 100 ml liposuction material was transferred to a blood bag (400 ml; Macopharma, Langen, Germany) and washed with an equal volume of phosphate-buffered saline (PBS) to remove blood and tumescence solution. Afterwards, for tissue digestion, PBS was replaced with 0.2 U/ml collagenase NB4 (Serva, Vienna, Austria) dissolved in 100 ml PBS containing Ca²⁺/Mg²⁺ and 25 mM N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid (HEPES; Sigma, Vienna, Austria) and the blood bag was incubated at 37 °C under moderate shaking (180 rpm) for 1 h. The digested tissue was transferred into 50 ml tubes. After centrifugation at 1200×g for 7 min the cell pellet was incubated with 100 ml erythrocyte lysis buffer [154 mM ammonium chloride (Sigma), 10 mM potassium



FIGURE 1 Extracorporeal shock wave therapy (ESWT) *in situ*. Seven patients were treated on their left side below the iliac crest 1 week and 1 h before liposuction with a Zimmer Z WavePro radial extracorporeal shock wave using two times 2000 pulses with 120 mJ/cm^2 and 16 Hz. The right side remained untreated (control). Afterwards, enzymatic isolation of the stromal vascular fraction (SVF) from liposuction waste material was performed

bicarbonate (Sigma), 0.1 mM ethylenediamine-tetraacetic acid (EDTA; Biochrom, Vienna, Austria) in aqua dest] for 3 min at 37°C to eliminate red blood cells. The supernatant was aspirated after centrifugation for 5 min at $500\times g$. The pellet was washed with PBS and filtered through a $100 \mu\text{m}$ cell strainer (Greiner, Kremsmünster, Austria). After another centrifugation step at $500\times g$ for 5 min the supernatant was removed and the isolated SVF cells were cultured at 37°C , 5% CO_2 and 95% air humidity in endothelial growth medium (EGM-2; Lonza, Vienna, Austria) containing 2% fetal bovine serum, hydrocortisone, human fibroblast growth factor, vascular endothelial growth factor (VEGF), R3-insulin-like growth factor-1 (R3-IGF-1), ascorbic acid, human epidermal growth factor (hEGF), GA-1000 and heparin or resuspended in EGM-2 for further analyses.

2.2 | Cell yield and viability

Cell number was determined using trypan blue exclusion and quantification in a cell counter (TC-20, Biorad, Vienna, Austria). For measuring cell viability, the percentage of living cells compared with the total cell count was analysed with the cell counter.

2.3 | Cellular ATP

To determine the cellular ATP concentration, a CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Mannheim, Germany) was

used and performed according to the manufacturer's instructions. Freshly isolated SVF cells were seeded at a density of 1×10^4 cells per well in a black 96-well plate (Greiner) in $100 \mu\text{l}$ EGM-2. After 2 h, $100 \mu\text{l}$ CellTiter-Glo® Reagent were added to each well and the plate was gently agitated on a shaker for 2 min. The plate was then incubated for 10 min in the dark. The luminescent signal was detected with an Infinite® M200 Multimode Microplate Reader (Tecan, Grödig, Austria) at an exposure time of 2 s and compared with an ATP standard curve.

2.4 | Proliferation

The proliferation potential was analysed by determining the population doubling level (PDL) with the following formulas:

$$k = \frac{\ln N - \ln N_0}{t_1 - t_0}$$

$$t_2 = \frac{\ln 2}{k}$$

$$\text{PDL} = \frac{t_1}{t_2}$$

where k is the growth constant, N_0 is the number of seeded cells, N is the number of cells after passing, t_0 is the time of seeding, t_1 is the time of passing and t_2 is the generation time.

Freshly isolated SVF cells were seeded at a density of 5×10^5 cells per T-25 culture flask in EGM-2 and the medium was changed every 3–4 days. When cells had reached a subconfluent state, they were passaged and the cell number was determined as described above. For the analysis of the PDL from passage 1 to 3, ASC were seeded at a density of 5×10^4 per T-25 culture flask and the cell number determined at each passage.

2.5 | Flow cytometry analysis

Freshly isolated SVF cells were characterized using the following antibodies: HLA-DR-PE (eBiosciences, Vienna, Austria), CD73-FITC (BD, Schwechat, Austria), CD90-PE (eBiosciences), CD105-PE (eBiosciences), CD14-FITC (BD), CD45-PerCP (BD), CD31-FITC (eBiosciences), CD34-APC (BD) and CD146-PerCP (R&D, Wiesbaden-Nordenstadt, Germany). For staining, 5×10^5 cells in 50 μ l PBS with 1% fetal calf serum (FCS; PAA, Pasching, Austria) were incubated with 5 μ l primary labelled antibodies at room temperature for 15 min in the dark. Cells were washed with 1.5 ml Cell Wash™ (BD) and centrifuged for 5 min at 400 \times g. The supernatant was discarded and the cell pellet resuspended in 300 μ l $1 \times$ Cell Fix™ (BD; diluted 1:10 with aqua dest). Samples were analysed on a FACSCalibur (BD).

2.6 | Adipogenic differentiation and detection

For adipogenic differentiation, ASC after 1 week in culture (passage 0) were plated at a density of 1.4×10^4 cells per well in a 24-well plate in EGM-2 and incubated overnight. The next day, the medium was changed to adipogenic differentiation medium [DMEM high glucose (Lonza) containing 10% FCS, 2 mM L-glutamine (PAA), 100 U/ml penicillin/streptomycin (Lonza), 1 μ M dexamethasone (Sigma), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma), 10 μ g/ml insulin (Sigma) and 100 μ M indomethacin (Sigma)] or control medium (DMEM:F12/L-glutamine (Lonza) with 10% FCS and 100 U/ml penicillin/streptomycin). The medium was changed every 3–4 days. After 21 days, adipogenic differentiation was analysed with Oil Red O staining and quantification. Cells were fixed with 4% formaldehyde for 1 h. After washing with aqua dest, the cells were rinsed with 70% ethanol for 2 min and stained for 5–15 min with Oil Red O solution (Sigma). The cells were then washed with aqua dest, counterstained for 1–3 min with haematoxylin solution and blued with tap water. For quantitative detection of Oil Red O staining, the supernatant was discarded and 500 μ l isopropanol were added to each well. After resuspension, the mixture of cells and isopropanol was transferred to a transparent 96-well plate (100 μ l per well). The absorbance was measured at 510 nm with an Infinite® M200 Multimode Microplate Reader.

2.7 | Osteogenic differentiation and detection

For osteogenic differentiation, ASC after 1 week in culture (passage 0) were seeded at a density of 2×10^3 cells per well in a 24-well plate in EGM-2 and incubated overnight. On the next day, medium was changed to osteogenic differentiation medium [DMEM low glucose (Lonza) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 10 nM dexamethasone, 150 μ M ascorbate-2-phosphate (Sigma), 10 mM β -glycerophosphate (StemCell Technologies, Cologne,

Germany) and 10 nM dihydroxy-vitamin D3 (Sigma)] or control medium [DMEM: F12/L-glutamine with 10% FCS and 100 U/ml penicillin/streptomycin]. The medium was changed every 3–4 days. After 21 days, osteogenic differentiation was analysed with Alizarin Red staining and quantification, as well as the determination of intracellular alkaline phosphatase (ALP) activity. For Alizarin Red staining of calcified structures, cells were fixed for 1 h with 70% ethanol at -20°C and stained with Alizarin Red solution (Merck, Vienna, Austria) for 15 min. For quantitative analysis of Alizarin Red staining, the supernatant was discarded and the cells were incubated with 500 μ l 20% methanol and 10% acidic acid (diluted in aqua dest) for 15 min. After resuspension, the mixture of cells and methanol/acidic acid was transferred to a transparent 96-well plate (100 μ l per well). The absorbance was measured at 450 nm with an Infinite® M200 Multimode Microplate Reader. The second method to analyse osteogenic differentiation was the detection of intracellular ALP activity. Cells were incubated with 100 μ l PBS for 1 h at -20°C . Cells were then lysed for 1 h by the addition of 100 μ l PBS containing 0.5% TritonX-100 (Sigma). For quantitative detection of ALP activity, 100 μ l substrate solution (4-nitrophenylphosphate) were added in each well and incubated for 1 h in the dark. Finally, the solution was transferred to a transparent 96-well plate (100 μ l per well) and absorbance was measured at 405 nm, together with a reference wavelength of 620 nm in an Infinite® M200 Multimode Microplate Reader. By creating a standard curve with known *p*-nitrophenol concentrations diluted in a solution containing 0.5% TritonX-100 diluted in PBS 1:2 and measuring the corresponding absorption, the ALP activity of the samples was calculated.

2.8 | Chondrogenic differentiation and detection

For chondrogenic differentiation in three-dimensional micromass pellet cultures, 3×10^5 ASC after 1 week in culture (passage 0) were centrifuged in chondrogenic differentiation medium [hMSC Chondro BulletKit (Lonza) containing 10 ng/ml BMP-6 (R&D) and 10 ng/ml TGF- β 3 (Lonza)] in screw cap microtubes. The tubes were placed in an incubator with slightly open caps for gas exchange. After 2 days, the pellets were transferred to 96-well U-bottom plates (Greiner) with fresh medium. The medium was changed every 2–3 days. After 35 days of differentiation, micromass pellets were fixed in 4% phosphate-buffered formalin overnight for histological analysis (Alcian Blue, collagen type II) (Oberbauer et al., 2016). The next day the pellets were washed in $1 \times$ PBS and dehydrated in increasing concentrations of alcohol. After rinsing the pellets in xylol and infiltration with paraffin, deparaffinized sections were stained with Alcian Blue for 30 min and counterstained for 2 min with haematoxylin. For collagen type II staining, sections were treated with pepsin for 10 minutes at 37°C (AP-9007 RTU, Thermo Scientific, Vienna, Austria). Endogenous peroxidase was quenched with freshly prepared 3% H_2O_2 for 10 min at room temperature, followed by normal horse serum 2.5% (Vector RTU) to block unspecific binding. Sections were incubated for 1 h with monoclonal anti-collagen type II (MS-306 P0 Thermo Scientific) at 1:100. After washing with Tris-buffered saline (TBS), sections were incubated with the secondary antibody (anti-mouse DAKO EnVision+ System horseradish peroxidase (HRP) labelled Polymer, Dako, Vienna,

Austria) for 30 min and rinsed in TBS again. Bindings were visualized using Nova Red (SK4800 Vector Labs, Vienna, Austria) for 6 min. Counterstaining was performed with haematoxylin for 2 min.

2.9 | Secretory profile

Freshly isolated SVF cells (5×10^5) were seeded in 2.5 ml EGM-2 in a T-25 flask for conditioning of the medium. After incubation for 2 h in EGM-2, the medium was changed to serum-free medium (DMEM low glucose with 2 mM L-glutamine). To also include non-adherent suspension cells, EGM-2 was withdrawn, collected and centrifuged at $300 \times g$ for 5 min to obtain a pellet of suspension cells. The supernatant was discarded after centrifugation and suspension cells were resolved in 500 μ l serum-free medium and returned to the original culture flask. The adherent cells were meanwhile provided with 2 ml serum-free medium. Twenty-four hours after seeding the supernatant was collected and stored at -80°C until analysis.

For the analysis of secreted angiogenic proteins, a Human Angiogenesis Array (RayBio, Georgia, USA) was used according to the manufacturer's instructions. Briefly, the membrane was blocked with a blocking buffer for 30 min prior to sample incubation for 5 h at room temperature. Every incubation and washing step excluding the incubation of detection buffer was performed under gentle rotation (0.8 cycles/s). After washing, the biotinylated antibody was incubated overnight at 4°C and membrane was washed again afterwards. HRP-conjugated streptavidin was incubated for 2 h at room temperature and membrane was washed again. Signals were detected by enhanced chemiluminescence and recorded on an X-ray

film (Figure S1). Signals were densitometrically quantified using ImageJ (NIH, Bethesda, MD, USA).

2.10 | Statistical analysis

Data are presented as box-plots (described as median in the text) or mean \pm standard deviation. A statistical analysis was performed using PRISM6 (GraphPad, San Diego, CA, USA), parametric two-tailed *t*-test or one-way ANOVA Tukey's post-hoc. *p*-values < 0.05 were considered to be significant.

3 | RESULTS

3.1 | Increased cell viability and proliferation after ESWT

Cells obtained from the untreated and ESWT-treated side were evaluated for their cell yield, viability and proliferation. After ESWT treatment, 5.2×10^5 cells were obtained compared with 4.6×10^5 cells from the untreated side (Figure 2a). The viability of the freshly isolated cells was significantly increased after ESWT treatment, with 88.4% living cells compared with 80.0% for the untreated side (Figure 2b). Similarly, ESWT treatment significantly enhanced the ATP concentration of freshly isolated cells from 331 nmol/l for the untreated side to 473 nmol/l for the ESWT-treated side (Figure 2c). Cells obtained from ESWT-treated and -untreated sides showed similar proliferation, calculated as PDL, after 7 days (1.8 ± 1.2 vs. 2.1 ± 1.1) and 14 days (7.3 ± 1.9 vs.

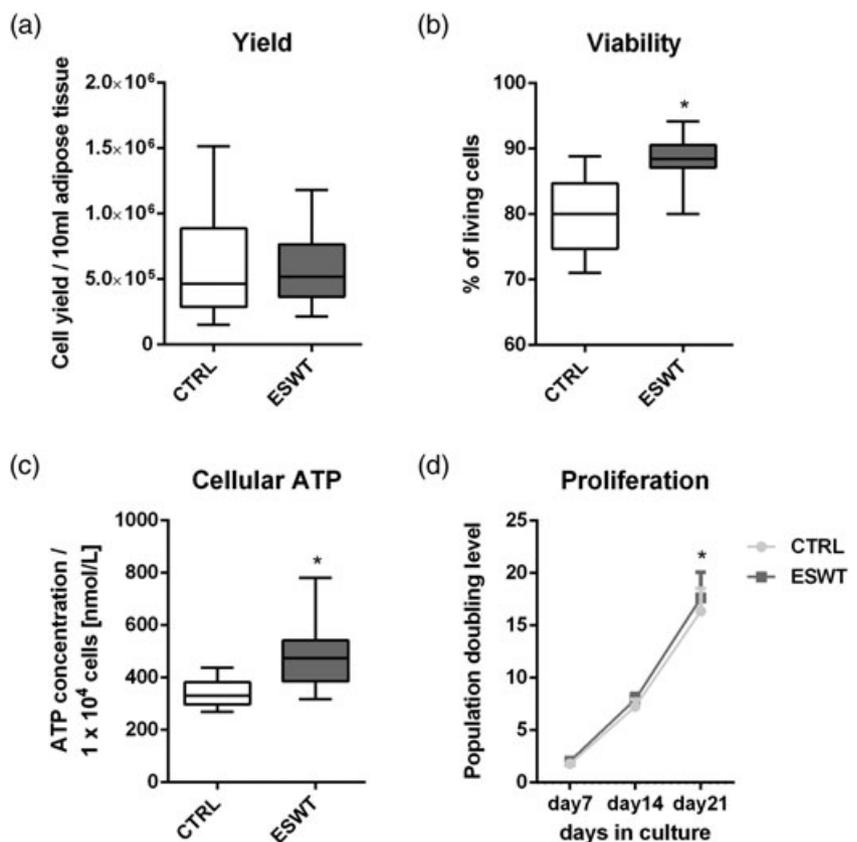


FIGURE 2 Extracorporeal shock wave therapy (ESWT) *in situ* maintains yield, enhances viability, adenosine triphosphate (ATP) concentration and proliferation of isolated cells. The cell yield was not affected by ESWT treatment (a), whereas there was a significant increase in viability (b) and ATP concentration (c). ESWT treatment enhanced the population doubling level of isolated cells after 21 days in culture (d). Data are presented as box-plots (a–c) or mean \pm standard deviation (d). **p* < 0.05

7.9 ± 1.6), but a significant increase after 21 days in culture (16.4 ± 6.2 vs. 17.6 ± 6.9) (Figure 2d).

3.2 | SVF surface marker expression after ESWT

For analysis of the cellular composition of the isolated SVF cells, the surface marker expression was investigated. ESWT treatment significantly

reduced the number of cells expressing the histocompatibility marker HLA-DR from 45.2% for the untreated side to 41.2% for the treated side (Figure 3). Expression of mesenchymal markers CD73, CD90 and CD105 was significantly increased after ESWT treatment (Figure 3). ESWT treatment resulted in 55.1% CD73-, 64.0% CD90- and 48.5% CD105-positive cells for the treated side compared with the untreated side with 29.4% CD73-, 44.8% CD90- and 21.8% CD105-positive cells.

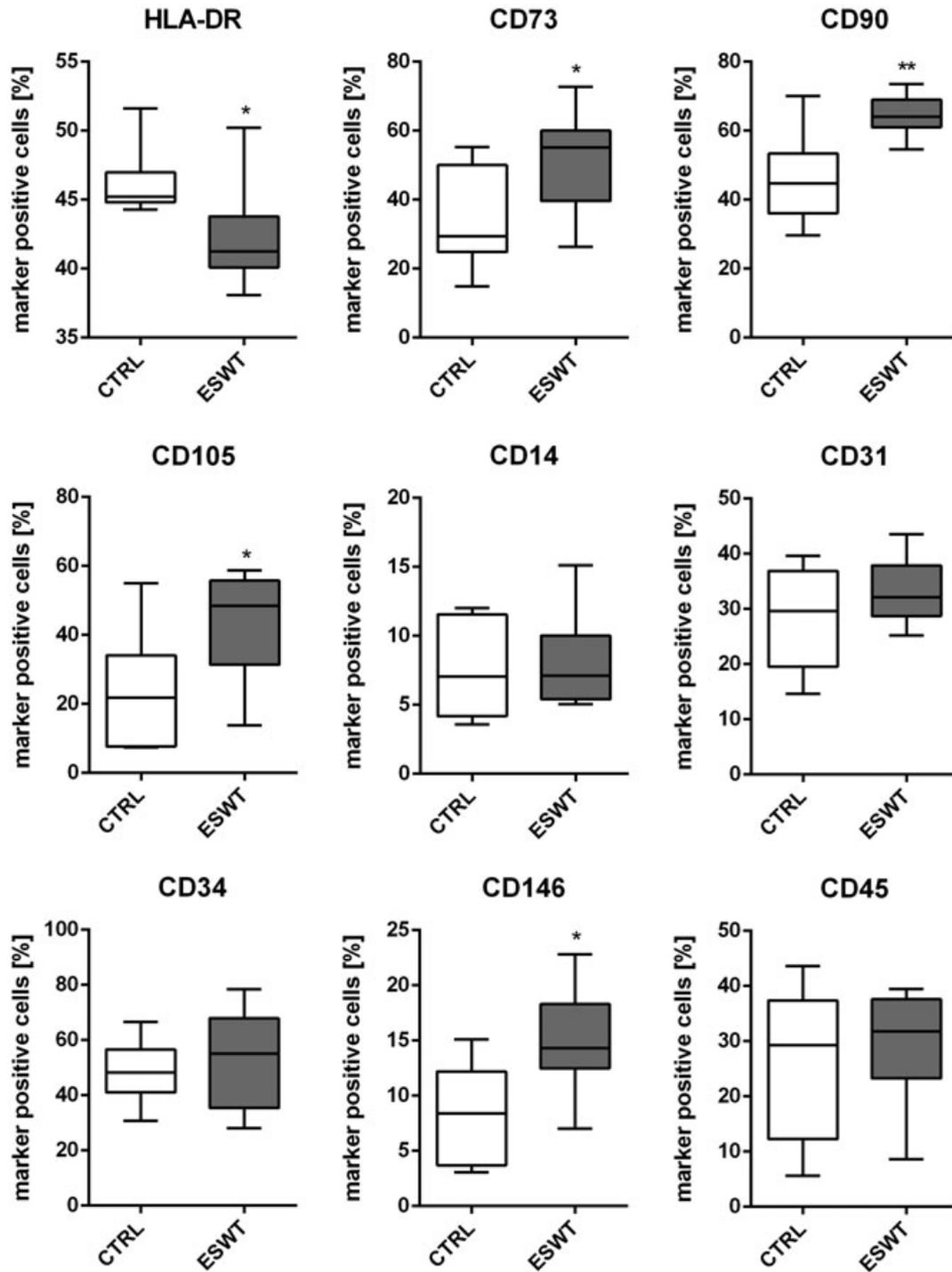


FIGURE 3 Extracorporeal shock wave therapy (ESWT) *in situ* positively affects the surface marker profile of isolated cells. After ESWT treatment there was a significant downregulation of the histocompatibility marker HLA-DR. The mesenchymal stem cell markers CD73, CD90 and CD105 were significantly enhanced after ESWT treatment. The monocyte/macrophage marker CD14, the endothelial marker CD31, the haematopoietic progenitor/vascular endothelial marker CD34 and the haematopoietic marker CD45 were not affected through ESWT treatment, whereas the expression of the endothelial/pericytic marker CD146 was significantly increased compared with the untreated side. Data are presented as box-plots. $n = 7$ for all tested markers except HLA-DR ($n = 4$). * $p < 0.05$, ** $p < 0.01$

ESWT treatment did not affect the number of cells positive for the monocyte/macrophage marker CD14, with 7.1% for both untreated and ESWT-treated sides (Figure 3). Also, expression of the endothelial marker CD31, the haematopoietic progenitor/vascular endothelial marker CD34 and the haematopoietic marker CD45 was not significantly affected after ESWT treatment (Figure 3). Cells derived from the ESWT-treated side showed an expression of 32.1% CD31, 55.2% CD34 and 31.7% CD45, whereas cells derived from the untreated side showed an expression of 29.7% CD31, 48.3% CD34 and 29.3% CD45. By contrast, the endothelial/pericytic marker CD146 was significantly increased after ESWT treatment, with an expression of 14.3% compared with 8.4% for cells obtained from the untreated side (Figure 3).

3.3 | Enhanced differentiation potential after ESWT

Adipose tissue-derived cells from untreated and ESWT-treated sides were analysed for their *in vitro* adipogenic, osteogenic and chondrogenic differentiation potential. Upon adipogenic induction, cells obtained from the ESWT-treated side clearly showed more lipid droplet formation, analysed by Oil Red O staining (Figure 4a, b). This observation was confirmed by quantitative analysis, which demonstrated a significant difference between the untreated side with an extinction of 0.9 and the ESWT-treated side with 1.2 (Figure 4c). Osteogenic differentiation potential analysed by Alizarin Red staining for mineralization was also slightly enhanced after ESWT treatment, which is reflected in the quantitative analysis with an extinction of 1.4 for the untreated side and 1.8 for the ESWT-treated side (Figure 5a–c). Similarly, ALP activity was increased upon osteogenic

induction after ESWT treatment, with 427 $\mu\text{mol/l}$ compared with the untreated side with 379 $\mu\text{mol/l}$ (Figure 5d). Moreover, cells derived from the ESWT-treated side demonstrated a stronger chondrogenic differentiation potential, illustrated by intense Alcian Blue (glycosaminoglycans) (Figure 6a, b) and collagen type II staining compared with the untreated side (Figure 6c, d). The diameter area of the three-dimensional micromass pellets was similar after 7 days ($1.1 \pm 0.1 \text{ mm}^2$ vs. $1.2 \pm 0.3 \text{ mm}^2$), 14 days ($1.0 \pm 0.1 \text{ mm}^2$ vs. $0.9 \pm 0.2 \text{ mm}^2$), 21 days ($0.9 \pm 0.2 \text{ mm}^2$ vs. $0.8 \pm 0.3 \text{ mm}^2$) and 28 days ($0.9 \pm 0.3 \text{ mm}^2$ vs. $1.0 \pm 0.6 \text{ mm}^2$), but slightly increased after 35 days of chondrogenic induction ($1.1 \pm 0.5 \text{ mm}^2$ vs. $1.3 \pm 1.0 \text{ mm}^2$) for cells obtained from the ESWT-treated side (Figure 6e).

3.4 | Secretory protein profile after ESWT

In order to explore angiogenic proteins that might be involved in the ESWT-induced changes, supernatants of SVF cells were collected 24 h after isolation and protein expression was analysed. ESWT treatment showed substantial changes in the secretion of angiogenesis-related proteins. Cells derived from the ESWT-treated site showed a significantly enhanced expression of interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and tissue inhibitor of metalloproteinase 1 (TIMP-1) (Figure 7). The expression of IL-6 was increased from 0.62- to 0.91-fold, MCP-1 from 0.74- to 0.93-fold and TIMP-1 from 0.47- to 0.68-fold. There was also a clear but not significant increase in the expression of EGF, IL-8, TIMP-2, urokinase-type plasminogen activator receptor (uPAR), IGF-1, leptin, thrombopoietin (TPO), VEGF-A and VEGF-D. All other analysed proteins did not show significant changes after ESWT treatment (Figure S2) and are summarized in Figure S1.

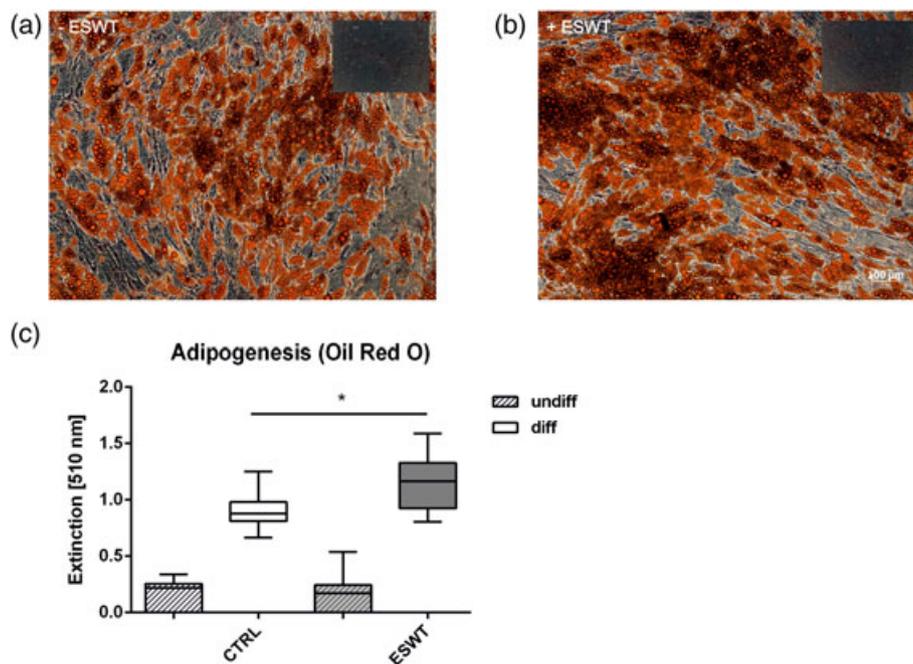


FIGURE 4 Extracorporeal shock wave therapy (ESWT) *in situ* increases the adipogenic differentiation potential of isolated cells. Adipogenic induction resulted in a stronger Oil Red O staining of cells obtained from the ESWT-treated side compared with the untreated side (a, b), which was confirmed through quantitative analysis (c). Images show representative pictures, small inserts show cells in control medium without growth factors and stimuli (a, b). Regarding quantitative analysis of differentiation, all conditions showed a significant enhancement of differentiated cells compared with undifferentiated cells (c). undiff, cells in control medium without growth factors and stimuli; diff, cells in differentiation medium. Data are presented as box-plots (c). $n = 4$ in triplicate. * $p < 0.05$

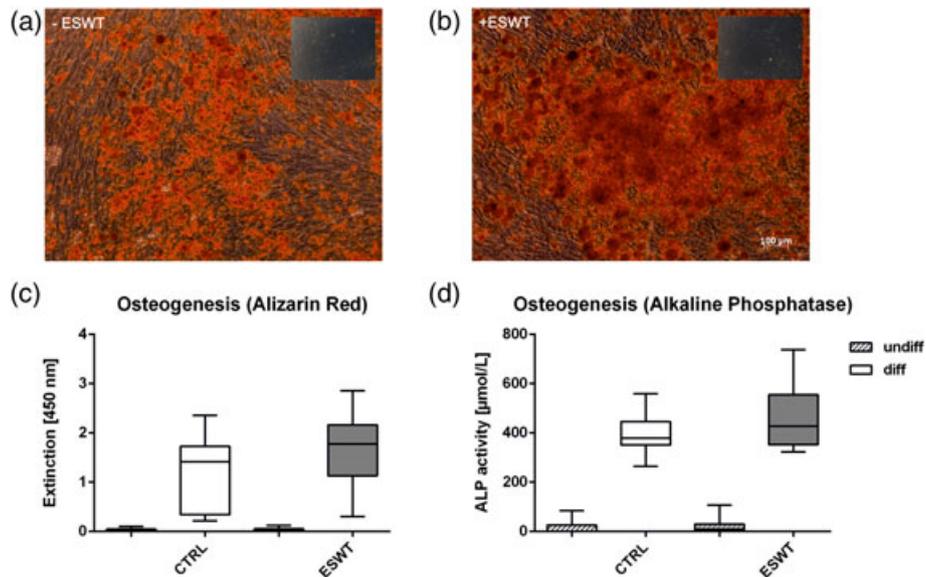


FIGURE 5 Extracorporeal shock wave therapy (ESWT) *in situ* maintains the osteogenic differentiation potential of isolated cells. Osteogenic differentiation analysed by Alizarin Red staining was slightly enhanced after ESWT treatment (a, b), which was confirmed through quantitative analysis of the staining (c) as well as ALP activity (d). Images show representative pictures, small inserts show cells in control medium without growth factors and stimuli (a, b). Regarding quantitative analysis of differentiation, all conditions showed a significant enhancement of differentiated cells compared with undifferentiated cells (c, d). undiff, cells in control medium without growth factors and stimuli; diff, cells in differentiation medium. Data are presented as box-plots (c, d). $n = 4$ in triplicate

4 | DISCUSSION

We have previously shown that the application of low-energy ESWT is promising for promoting stem cell properties in isolated cells derived from adipose tissue (Schuh et al., 2014; Schuh et al., 2016). Hence, the effect of ESWT applied to adipose tissue *in situ* on the regenerative cell populations present within their tissue microenvironment is worthwhile investigating.

In the present study we focused on the cell properties and functionality after shock wave treatment directly on the harvesting site in patients. Here we could demonstrate that ESWT applied *in situ* 1 week and 1 h before harvest resulted in significantly enhanced cell viability and ATP content in the isolated cell population, although the total cell yield remained unaffected. In a previous study, we demonstrated that ESWT on explanted rat sciatic nerves before isolation of Schwann cells significantly increased extracellular ATP. Subsequently, a number of effects were observed in culture: higher Schwann cell yield, higher cell purity and increased proliferation rate (Schuh et al., 2016). The release of ATP is triggered by ESWT, which could be proven in murine mesenchymal progenitor cells, primary human ASC and a human Jurkat T cell line *in vitro*. ATP release subsequently activates purinergic receptors and finally enhances proliferation via downstream Erk1/2 signalling (Weihs et al., 2014). ESWT-treated equine ASC showed increased proliferation, expression of Cx43, a gap junction protein and significant activation of Erk1/2, whereas no significant effect on the differentiation potential was observed (Raabe et al., 2013). Mitogen-activated protein kinases, including Erk1/2, regulate the stimulation of biophysical ESWT, thereby triggering mitogenic and osteogenic responses (Chen et al., 2004).

The proliferative activity of rat endothelial progenitor cells was optimally supported when cells were treated with ESWT within the

range of 0.10–0.13 mJ/mm² and 200–300 impulses, whereas higher-energy ESWT led to cell apoptosis (Zhang, Yan, Wang, Tang, & Chai, 2014). However, others have observed that ESWT with an energy intensity of 0.0016 mJ/mm² (at 4 kV) and 2000 pulses is able to disrupt pig adipose tissue (Liang, Chang, & Yang, 2014). Interestingly, we could corroborate an increase in proliferative potential after ESWT *in situ* with significant enhancement after 21 days in culture using an energy level of 120 mJ/cm² and two times 2000 impulses. The different outcomes can be explained by the type of shock waves (electrohydraulic, piezoelectric, electromagnetic, radial), applied energy (focused or unfocused, dose-dependent), the field of application (*in vitro*, *in vivo*, *in situ*) and the frequency of application (Ke et al., 2016; Lohrer, Nauck, Dorn-Lange, Scholl, & Vester, 2010; Lohrer, Nauck, Korakakis, & Malliaropoulos, 2016).

Furthermore, our ESWT *in situ* setting might activate stem and progenitor cells within their niches, leading to a significant higher amount of isolated cell populations expressing mesenchymal and endothelial/pericytic marker, while downregulating HLA-DR. In our previous study, ESWT in culture led to maintenance and significant elevation of mesenchymal markers (CD73, CD90, CD105) in human and rat ASC, accompanied by significantly increased differentiation capacity towards the osteogenic and adipogenic lineage as well as towards Schwann-cell like cells even after extended passaging, thus preserving multipotency of ASC *in vitro* (Schuh et al., 2014).

In this study, ESWT *in situ* increased significantly the adipogenic differentiation potential of isolated cells while osteogenic and chondrogenic differentiation was slightly enhanced. As the differentiation potential was analysed with a defined cell number, the adipogenic potential might not correlate to the cell yield obtained after isolation. This could, however, support the assumption that ESWT *in situ* targets a distinct subpopulation highly susceptible for adipogenic induction.

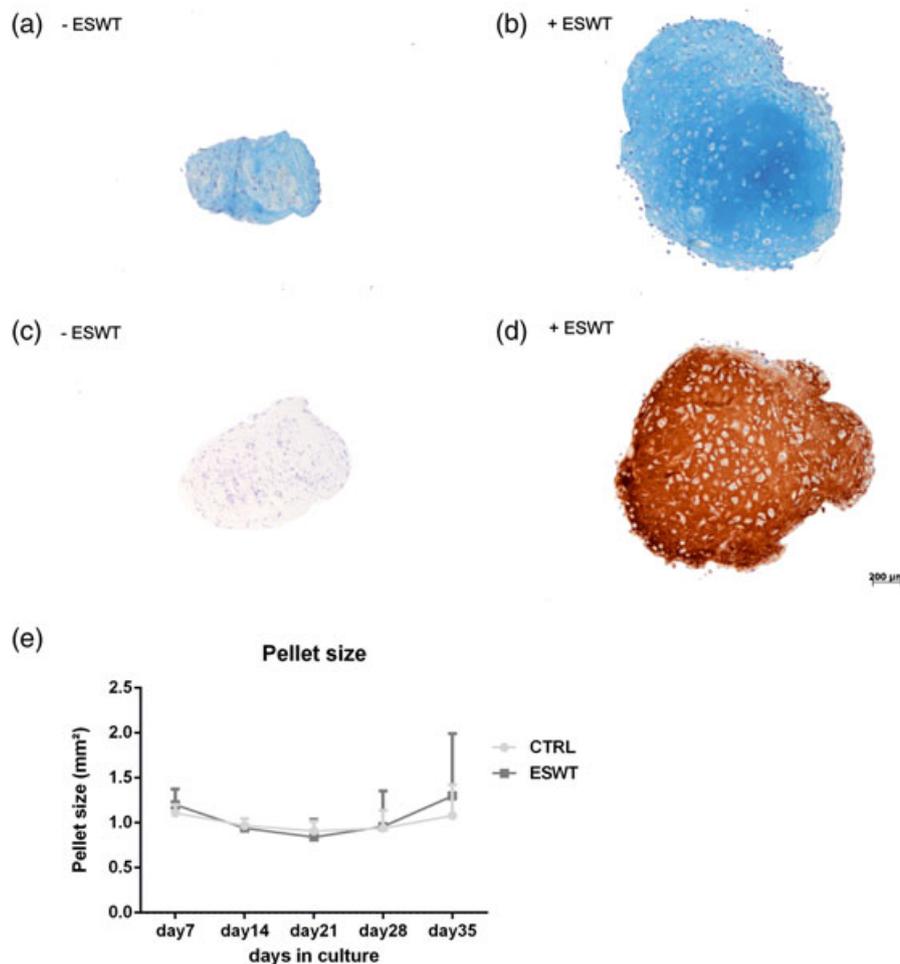


FIGURE 6 Extracorporeal shock wave therapy (ESWT) *in situ* increases the chondrogenic differentiation potential of isolated cells. ESWT treatment increased chondrogenic differentiation resulting in stronger staining of Alcian Blue (a, b) and collagen type II (c, d) after 35 days, as well as a slightly increased diameter area of the three-dimensional micromass pellets after 35 days of chondrogenic induction (e). Images show representative pictures (a–d). Data are presented as mean \pm standard deviation (e). $n = 3$

Dissimilar properties have been shown for ESWT-treated bone marrow mesenchymal stromal/stem cells (BMSC) from patients with avascular necrosis of the femoral head with induced osteoblast differentiation and concurrently inhibited adipogenic differentiation (Zhai et al., 2016). ESWT has also been shown to induce osteogenic activity of MG-63 cells seeded onto glass-ceramic scaffolds mediated by increased expression of bone morphogenetic proteins (Muzio et al., 2014). Apart from the mesodermal trilineage differentiation, ESWT treatment led to significantly accelerated human tendon-derived stem/progenitor cell differentiation *in vitro* (Leone et al., 2016).

A prerequisite in fat grafting is an early and abundant vascularization for nutrition supply and integration inside the surrounding tissue (Garza et al., 2014; Luo et al., 2013). ESWT may play a critical role in this manner as demonstrated in a previous study with enhanced lymphangiogenesis (Rohringer et al., 2014). ESWT induced VEGF expression in human umbilical vein endothelial cells (Holfeld et al., 2014). VEGF was secreted to culture medium and enhanced endothelial cell proliferation in an autocrine manner (Peng et al., 2015). Proliferation of rat BMSC and secretion of growth factors playing a role in regeneration settings as well as promotion of angiogenesis and nerve regeneration *in vitro* were enhanced by the application of ESWT (Zhao et al., 2013). In the present study we could

detect a substantial influence of ESWT treatment on the secretion of specific angiogenic proteins, such as IL-6, MCP-1 and TIMP-1. There was also a clear, although not significant, increase in the expression of EGF, IL-8, TIMP-2, uPAR, IGF-1, leptin, TPO, VEGF-A and VEGF-D. These factors might also have a paracrine impact on adipogenic differentiation of ASC. The pro-adipogenic impact of IGF-1 in SVF populations has been demonstrated previously (Hu et al., 2015). Leptin and IL-6 are also important markers in adipocyte differentiation (Vicennati, Vottero, Friedman, & Papanicolaou, 2002).

Within this study we could demonstrate the positive effect of ESWT applied *in situ* leading to enhanced cell properties, adipogenesis and angiogenesis *in vitro*. This set-up could reflect a clinical situation of a one-step procedure, avoiding risk factors such as a water bath, which is the prerequisite for ESWT *in vitro*, tissue processing, which requires a sterile workbench, collagenase, which might have a negative impact on cell efficacy, expansion of cells (ASC), which would be classified as advanced therapy medicinal product and would require central market authorization associated with high costs and stricter conditions. Conclusively, the use of ESWT *in situ* might be a promising tool to enhance the quality of fat grafts and in consequence a successful transplantation with long-term graft survival.

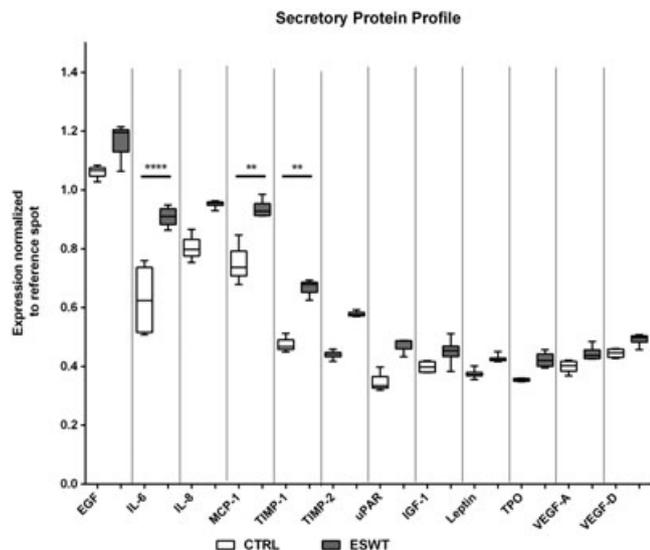


FIGURE 7 Extracorporeal shock wave therapy (ESWT) *in situ* changes the secreted angiogenic protein profile of isolated cells. ESWT treatment showed substantial changes in the expression of angiogenesis-related proteins. Three analysed proteins demonstrated a significantly enhanced expression after ESWT treatment: interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and tissue inhibitor of metalloproteinase 1 (TIMP-1). The expression of the following proteins was clearly but not significantly increased after ESWT: epidermal growth factor (EGF), IL-8, TIMP-2, urokinase-type plasminogen activator receptor (uPAR), insulin-like growth factor 1 (IGF-1), leptin, thrombopoietin (TPO), vascular endothelial growth factor A (VEGF-A) and VEGF-D. Data are presented as box-plots and represent duplicate measurements of two independent donors. ** $p < 0.01$, **** $p < 0.0001$

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CONFLICT OF INTEREST

We exclude any conflict of interest and guarantee that all authors are in complete agreement with the contents and submission of this manuscript. Furthermore, we declare that our work is original research, unpublished and not submitted to another journal.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. Overview of angiogenesis array membranes. Membranes include 43 different angiogenesis-related proteins, which are spotted in duplicates. Membranes were exposed to X-ray films and show different spot densities (a). Allocation of proteins throughout the membranes (b)

Figure S2. Angiogenic protein profiles of the angiogenesis array. ESWT treatment showed no significant changes in the expression of these angiogenesis-related proteins. Data are presented as box-plots and represent duplicate measurements of two independent donors

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