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

Short Title: Extracorporeal shock wave therapy activated fat grafts

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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, ATP content, proliferative capacity, surface marker profile, differentiation potential and secretory protein profile. While 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 to 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.

Key words

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

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 sagging of structures and formation of wrinkles (Amar and Fox, 2011; Sandhofer and Schauer, 2015). Hence, restoring volume by 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 and Katzel, 2015).

Autologous fat grafting has been described for the first time in 1893 by Gustav A. Neuber (Neuber, 1893). Nowadays, it experiences a comeback in cosmetic and plastic surgery due to its relative ease of application associated with comparatively low risks (Gir et al., 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 et al., 2009; Gentile et al., 2012; Jiang et al., 2015; Luo et al., 2013). Although fat grafting has been constantly further 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 following principles: gentle harvesting of fat to preserve the adipose tissue structure, centrifugation to get rid of nonviable components and to concentrate the fat, and

delivery of adipose tissue in small aliquots to facilitate adequate blood supply and thus maximizing graft take and graft retention of up to 65% (Coleman and Katzel, 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 et al., 2015). Furthermore, the main factor influencing long-term graft survival is still a matter of debate. Whereas older studies concentrate on viability of mature adipocytes in the graft there has been a shift towards 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., 2015).

In 2006, a novel approach, known as cell assisted lipotransfer (CAL), has been 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 CAL can reduce postoperative atrophy in breast augmentation (Salgarello et al., 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). Since cell properties might change with isolation procedure, the comparison of the outcome of clinical trials may be tricky. 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 has been 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 and Reddy, 2011). However, this technology has also emerged as an effective noninvasive treatment approach for several other indications including musculoskeletal disorders such as tendinopathies and bone defects (delayed-/non-union of bone fractures, avascular necrosis of femoral head) (Saggini et al., 2008), problematic soft tissue wounds (Antonic et al., 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 treating patients with cellulite, lipedema and lymphedema (Cebicci et al., 2016; Sandhofer, 2015; Siems et al., 2005). In cellulite and lipedema patients, ESWT significantly improved the biomechanic skin properties leading to smoothening of dermis and hypodermis surface (Siems et al., 2005). This effect is amongst others caused by remodeling of collagen in the ESWT treated regions (Angehrn et al., 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 adenosinetriphosphate (ATP) release that subsequently binds to purinergic receptors acting via downstream Erk1/2 signaling on cell proliferation (Chen et al., 2004; Weihs et al., 2014). Besides increased growth potential also enhanced differentiation from progenitors into mature cell types has been observed (Chen et al., 2004; Leone et al., 2016; Muzio et al., 2014; Schuh et al., 2016; Zhai et al., 2016). In contrast, Raabe and co-workers did not find an effect of ESWT on the differentiation potential of equine ASC (Raabe et al., 2013). 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 thereof which might also be crucial for realizing cellular therapies (Schuh et al., 2016).

For this study, 7 female patients undergoing routine liposuction were treated with ESWT before the actual procedure. Subsequently, from the liposuction material the SVF was isolated and analyzed 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 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 extracorporeal shock wave therapy (ESWT) in situ

7 female donors were treated locally below the iliac crest one week and one hour before liposuction with radial ESWT (Z WavePro, Zimmer, Austria) using two doses of 2000 pulses with 120mJ/cm^2 and 16Hz (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, Austria) 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, Austria) dissolved in 100 ml PBS containing Ca^{2+}/Mg^{2+} and 25 mM N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid (HEPES; Sigma, Austria) and the blood bag was incubated at 37°C under moderate shaking (180 rpm) for 1 hour. The digested tissue was transferred into 50 ml-tubes. After centrifugation at 1200 g for 7 minutes the cell pellet was incubated with 100 ml erythrocyte lysis buffer (154 mM ammonium chloride (Sigma), 10 mM potassium bicarbonate (Sigma), 0.1 mM ethylenediamine-tetraacetic acid (EDTA; Biochrom, Austria) in aqua dest) for 3 minutes at 37°C to eliminate red blood cells. The supernatant was aspirated after centrifugation for 5 minutes at 500 g. The pellet was washed with PBS and filtered through a 100-µm cell strainer (Greiner, Austria). After another centrifugation step at 500 g for 5 minutes 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, Austria) containing 2% fetal bovine serum (FBS), hydrocortisone, human fibroblastic growth factor (hFGF), 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). For measuring cell viability, the percentage of living cells compared to total cell count was analyzed with the cell counter.

2.3. Cellular ATP

To determine the cellular ATP concentration CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Austria) was used and performed according to the manufacturer's instructions. Freshly isolated SVF cells were seeded at a density of $1x10^4$ cells per well in a black 96-well plate (Greiner) in 100 µL EGM-2 medium. After 2 hours, 100 µL CellTiter-Glo® Reagent were added to each well and the plate was gently agitated on a shaker for 2 minutes. Afterwards the plate was incubated for 10 minutes in the dark. The luminescent signal was detected with an Infinite® M200 Multimode Microplate Reader (Tecan, Austria) at an exposure time of 2 seconds and compared with an ATP standard curve.

2.4. Proliferation

Proliferation potential was analyzed by determining the population doubling level (PDL) with the following formulas:

$$k = \frac{\ln N - \ln N0}{t1 - t0}$$

$$t2 = \frac{\ln 2}{k}$$

$$PDL = \frac{t1}{t2}$$

k: growth constant, N0: amount of seeded cells, N: amount of cells after passaging, t0: time of seeding, t1: time of passaging, t2: generation time

Freshly isolated SVF cells were seeded at a density of $5x10^5$ cells per T-25 culture flask in EGM-2 and medium was changed every 3 to 4 days. When cells had reached a subconfluent state, they were passaged and cell number was determined as described above. For analysis of PDL from passage 1 to 3, ASC were seeded at a density of $5x10^4$ per T-25 culture flask and 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, Austria), CD73-FITC (BD, Austria), CD90-PE (eBiosciences), CD105-PE (eBiosciences), CD14-FITC (BD), CD45-PerCP (BD), CD31-FITC (eBiosciences), CD34-APC (BD) and CD146-PerCP (R&D, Austria). For staining, $5x10^5$ cells in 50 µL PBS with 1% fetal calf serum (FCS; PAA, Austria) were incubated with 5 µl primary labeled antibodies at room temperature for 15 minutes in the dark. Cells were washed with 1.5 ml Cell Wash™ (BD) and centrifuged for 5 minutes at 400 g. The supernatant was discarded and the cell pellet resuspended in

300 µL 1 x Cell Fix™ (BD; diluted 1:10 with aqua dest). Samples were analyzed on a FACSCalibur (BD).

2.6. Adipogenic differentiation and detection

For adipogenic differentiation, ASC after one week in culture (passage 0) were plated at a density of $1.4x10^4$ cells per well in a 24-well plate in EGM-2 medium and incubated over night. On the next day, medium was changed to adipogenic differentiation medium (DMEM-high glucose (Lonza) containing 10% FCS, 2 mM L-glutamine, 100 U/ml Pen/Strep, 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma), 10 µg/ml insulin (Sigma) and 100 µM indomethacin (Sigma)) or control medium (DMEM:F12 / L-glutamine with 10% FCS and 100 U/ml Pen/Strep). Medium was changed every 3 to 4 days. After 21 days, adipogenic differentiation was analyzed with Oil Red O staining and quantification. Cells were fixed with 4% formaldehyde for 1 hour. After washing with aqua dest, the cells were rinsed with 70% ethanol for 2 minutes and stained for 5-15 minutes with Oil Red O solution (Sigma). Then the cells were washed with aqua dest, counterstained for 1-3 minutes with hematoxylin solution and blued with tap water. For quantitative detection of Oil Red O staining, the supernatant was discarded and 500 µl isopropanol were added in each well. After resuspension, the mixture of cells and isopropanol was transferred to a transparent 96-well plate (100 µl 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 one week in culture (passage 0) were seeded at a density of $2x10^3$ cells per well in a 24-well plate in EGM-2 and incubated over night. On the next day, medium was changed to osteogenic differentiation medium (DMEM-low glucose (Lonza) containing 10% FCS, 2 mM L-glutamine (PAA), 100 U/ml Pen/Strep (Lonza), 10 nM dexamethasone (Sigma), 150 µM ascorbat-2-phosphate (Sigma), 10 mM β-glycerophosphate (StemCell Technologies, Germany) and 10 nM dihydroxy-vitamin D3 (Sigma)) or control medium (DMEM:F12 / L-glutamine (Lonza) with 10% FCS and 100 U/ml Pen/Strep). Medium was changed every 3 to 4 days. After 21 days, osteogenic differentiation was analyzed with Alizarin Red staining and quantification, as well as determination of intracellular alkaline phosphatase (ALP) activity. For Alizarin Red staining of calcified structures, cells were fixed for 1 hour with 70% ethanol at -20°C and stained with Alizarin Red solution (Merck, Austria) for 15 minutes. For quantitative analysis of Alizarin Red staining, the supernatant was discarded and the cells were incubated with 500 µl 20% methanol and 10% acidic acid (diluted in aqua dest) for 15 minutes. After resuspension, the mixture of cells and methanol / acidic acid was transferred to a transparent 96-well plate (100 µl per well). The absorbance was measured at 450 nm with an Infinite® M200 Multimode Microplate Reader. The second method to analyze osteogenic differentiation is the detection of the activity of intracellular ALP. Cells were incubated with 100 µl PBS for 1 hour at -20°C. Afterwards, the cells were lysed for 1 hour by addition of 100 µl PBS containing 0.5% TritonX-100 (Sigma). For quantitative detection of ALP activity, 100 µl substrate solution (4-nitrophenylphosphate) were added in each well and incubated for 1 hour in the dark. Finally, the solution was transferred to a transparent 96-well plate (100 µl 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 3D micromass pellet cultures, $3x10^5$ ASC after one 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-ß3 (Lonza)) in screw cap micro tubes. 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. Medium was changed every 2 to 3 days. After 35 days of differentiation, micromass pellets were fixed in 4% phosphate-buffered formalin over night for histological analysis (Alcian Blue, collagen type II) (Oberbauer et al., 2016). The next day the pellets were washed in 1x 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 minutes and counterstained for 2 minutes with hematoxylin. For collagen type II staining, sections were treated with pepsin for 10 minutes at 37°C (AP-9007 RTU, Thermo Scientific, Austria). Endogenous peroxidase was quenched with freshly prepared 3% H_2O_2 for 10 minutes at room temperature, followed by normal horse serum 2.5% (Vector RTU) to block unspecific binding. Sections were incubated 1 hour with monoclonal anti-collagen type II (MS-306 P0 Thermo Scientific) at 1:100. After washing with TBS, sections were incubated with

the secondary antibody (anti mouse DAKO EnVision+ System HRP labelled Polymer, Dako, Austria) for 30 minutes and rinsed in TBS again. Bindings were visualized using Nova Red (SK4800 Vector Labs, Austria) for 6 minutes. Counterstaining was performed with hematoxylin for 2 minutes.

2.9. Secretory profile

Freshly isolated SVF cells ($5x10^5$) were seeded in 2.5 ml EGM-2 in a T-25 flask for conditioning of the medium. After incubation for 2 hours in EGM-2 medium was changed to serum-free medium (DMEM-low glucose with 2mM L-glutamine). To include also non-adherent suspension cells EGM-2 was withdrawn, collected and centrifuged at 300 g for 5 minutes to obtain a pellet of suspension cells. The supernatant was discarded after centrifugation and suspension cells were resolved in 500 µl serum-free medium and returned to the original culture flask. The adherent cells were meanwhile provided with 2 ml serum-free medium. 24 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 minutes prior to sample incubation for 5 hours at room temperature. Every incubation and washing step excluding the incubation of detection buffer was performed under gentle rotation (0.8 cycles/sec). After washing, the biotinylated antibody was incubated over night at 4°C and membrane was washed again afterwards. HRP streptavidin was incubated for 2 hours at room temperature and membrane was washed again. Signals were detected by enhanced chemiluminescence and recorded on an X-ray film

(Supplemental Figure 1). Signals were densitometrically quantified using ImageJ (NIH, Bethesda, Maryland).

2.10. Statistical Analysis

Data are presented as box-plots (described as median in the text) or mean ± SD. 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 of < 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 $x10^5$ cells were obtained compared to 4.6 $x10^5$ cells from the untreated side (Figure 2a). Viability of the freshly isolated cells was significantly increased after ESWT treatment with 88.4% living cells compared to 80.0% for the untreated side (Figure 2b). Similarly, ESWT treatment significantly enhanced 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. 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 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 to the untreated side with 29.4% CD73, 44.8% CD90 and 21.8% CD105 positive cells.

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 hematopoietic progenitor/vascular endothelial marker CD34 and the hematopoietic 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, while cells derived from the untreated side showed an expression of 29.7% CD31, 48.3% CD34 and 29.3% CD45. In contrast, the endothelial/pericytic marker CD146 was significantly increased after ESWT treatment with an expression of 14.3% compared to 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 analyzed for their in vitro adipogenic, osteogenic and chondrogenic differentiation potential. Upon adipogenic induction, cells obtained from the ESWT treated side showed clearly more lipid droplet formation analyzed 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 ESWT treated side with 1.2 (Figure 4c). Osteogenic differentiation potential analyzed 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 µmol/l compared to the untreated side with 379 µmol/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 to the untreated side (Figure 6c, d). Diameter area of the 3D micromass pellets was similar after 7 days (1.1 ± 0.1 mm^2 vs 1.2 ± 0.3 mm^2), 14 days (1.0 ± 0.1 mm^2 vs 0.9 ± 0.2 mm^2), 21 days (0.9 ± 0.2 mm^2 vs 0.8 ± 0.3 mm^2) and 28 days (0.9 ± 0.3 mm^2 vs 1.0 ± 0.6 mm^2) but slightly increased after 35 days chondrogenic induction (1.1 ± 0.5 mm^2 vs 1.3 ± 1.0 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 which might be involved in the ESWT induced changes supernatants of SVF cells were collected 24 hours after isolation and protein expression was analyzed. 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 chemotactic 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 epidermal growth factor (EGF), interleukin-8 (IL-8), tissue inhibitor of metalloproteinase 2 (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 vascular endothelial growth factor D (VEGF-D). All other analyzed proteins did not show significant changes after ESWT treatment (Supplemental Figure 2) and are summarized in supplemental figure 1.

4. Discussion

We have previously shown that 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 on adipose tissue in situ on the regenerative cell populations present within their tissue microenvironment is worthwhile investigating.

We focused in the present study 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 one week and one hour pre 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 signaling (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 (MAPK) 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/mm^2 and 200-300 impulses whereas higher energy ESWT led to cell apoptosis (Zhang et al., 2014). However, others have observed that ESWT with an energy intensity of 0.0016 mJ/mm^2 (at 4 kV) and 2000 pulses is able to disrupt pig adipose tissue (Liang et al., 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/cm^2 and 2 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 et al., 2010; Lohrer et al., 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 toward 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. Since the differentiation potential was analyzed 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. 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). Besides the mesodermal trilineage differentiation, ESWT treatment led to significantly accelerated human tendon-derived stem/progenitor cells 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 treated (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 was enhanced by 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 proadipogenic 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 et al., 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 negative impact on cell efficacy, expansion of cells (ASC) which would be classified as ATMP 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.

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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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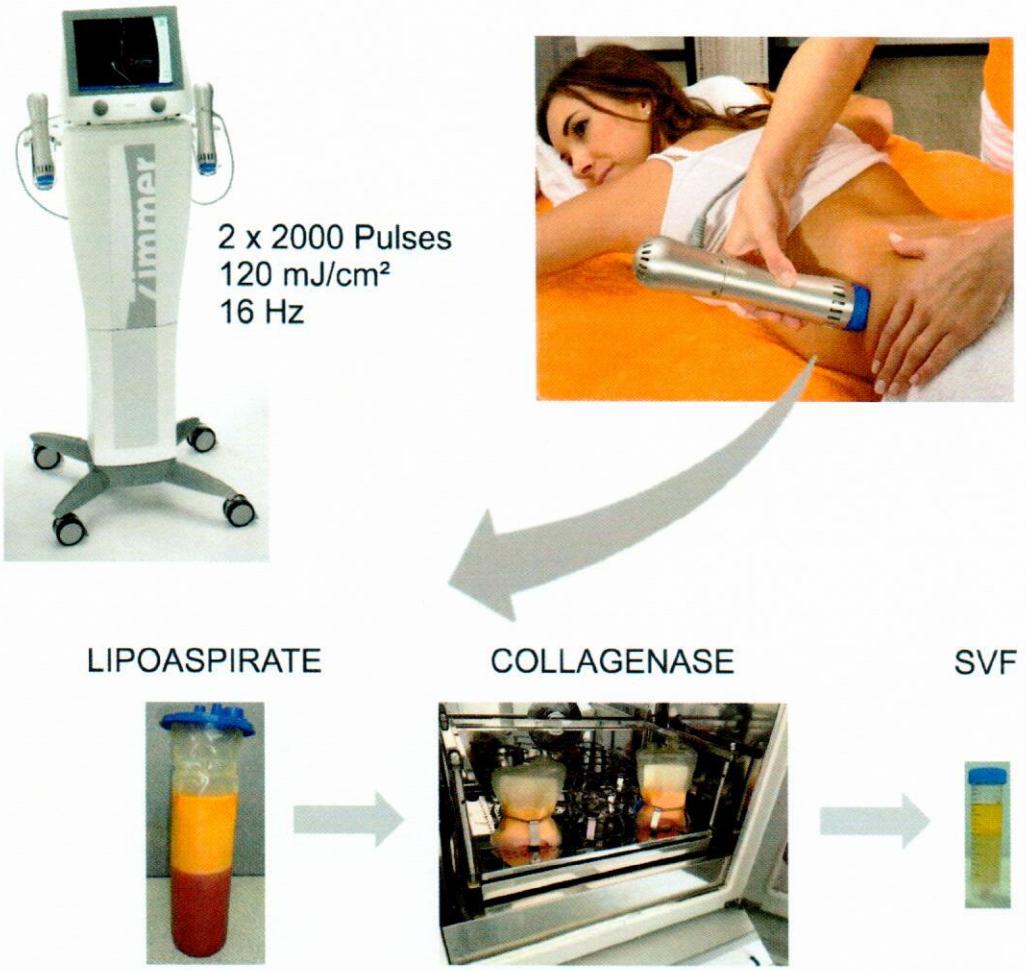

Figure 1. Extracorporeal shock wave therapy (ESWT) in situ. 7 Patients are treated on their left side below the iliac crest one week and one hour before liposuction with a Zimmer Z WavePro radial extracorporeal shock wave using two times 2000 pulses with 120mJ/cm^2 and 16Hz. The right side remained untreated (control). Afterwards, enzymatic isolation of SVF from liposuction waste material was performed.

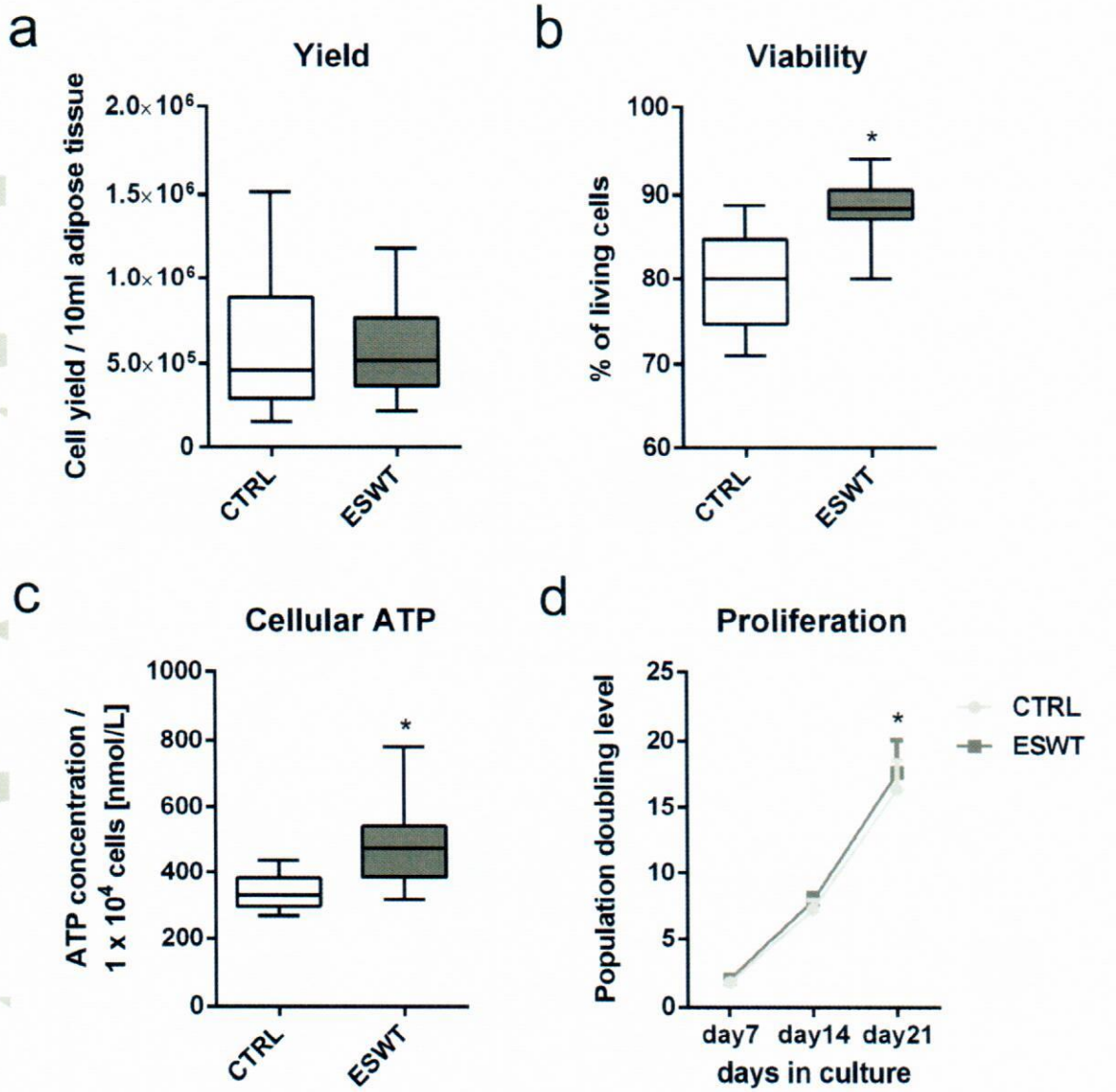

Figure 2. Extracorporeal shock wave therapy (ESWT) in situ maintains yield, enhances viability, ATP concentration and proliferation of isolated cells. The cell yield was not affected by ESWT treatment (a), while there was a significant increase in viability (b) and ATP concentration (c). ESWT treatment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

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 hematopoietic progenitor/vascular endothelial marker CD34 and the hematopoietic marker CD45 were not affected through ESWT treatment, while expression of the endothelial/pericytic marker CD146 was significantly increased compared to the untreated side. Data are presented as box-plots. n=7 for all tested markers except HLA-DR n=4. *$p < 0.05$, **$p < 0.01$.

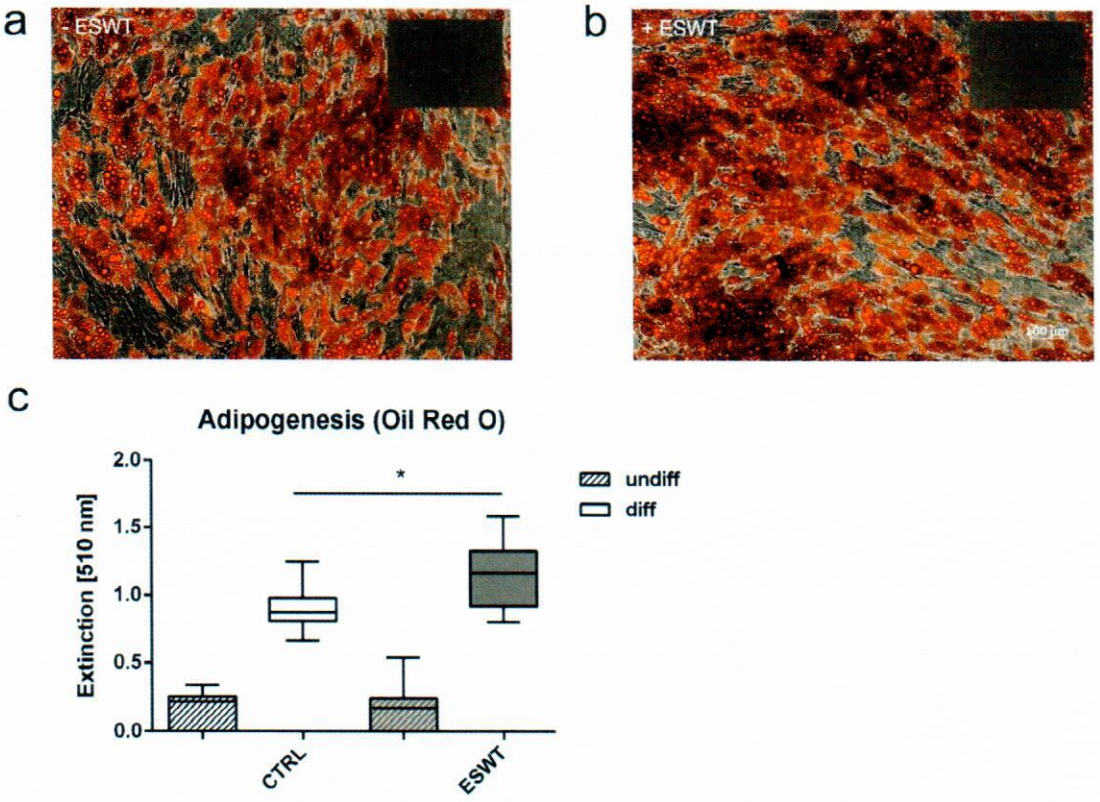

Figure 4. Extracorporeal shock wave therapy (ESWT) in situ increases the adipogenic differentiation potential of isolated cells. Adipogenic induction resulted in a stronger Oil Red O staining of cells obtained from the ESWT treated side compared to the untreated side (a, b), which was confirmed through 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 to 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. *$p < 0.05$.

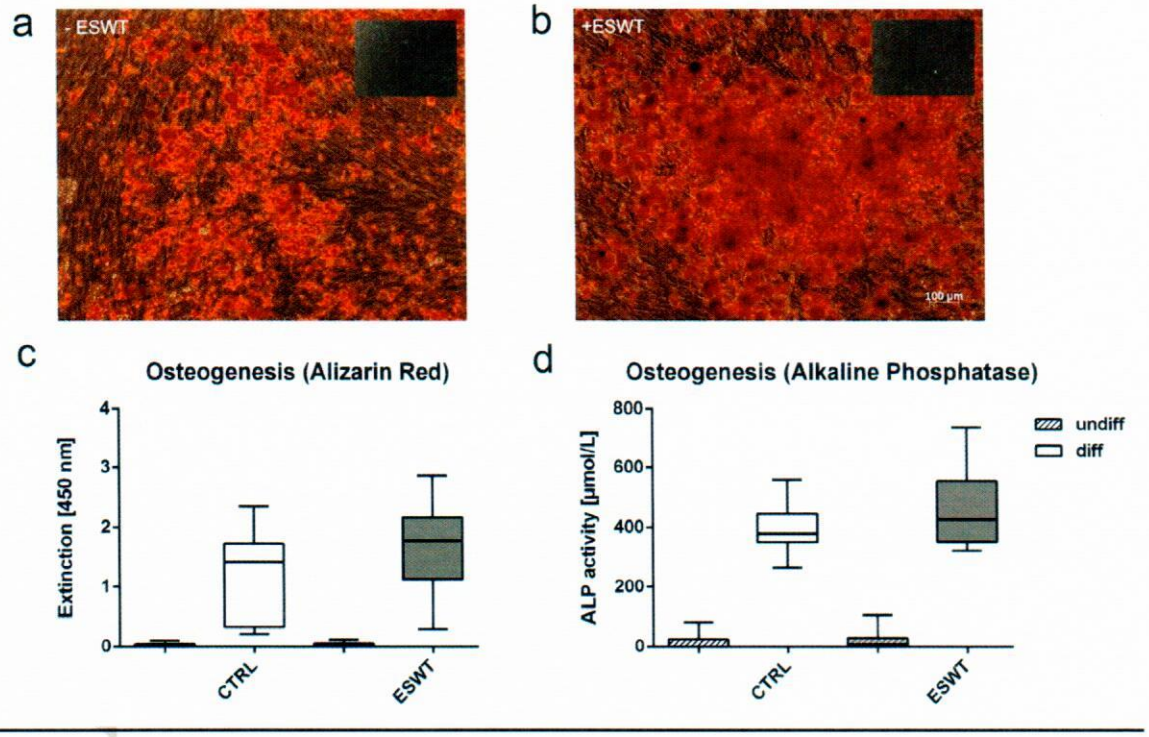

Figure 5. Extracorporeal shock wave therapy (ESWT) in situ maintains the osteogenic differentiation potential of isolated cells. Osteogenic differentiation analyzed by Alizarin Red staining was slightly enhanced after ESWT treatment (a, b), which was confirmed through quantitative analysis of the staining (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 to 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.

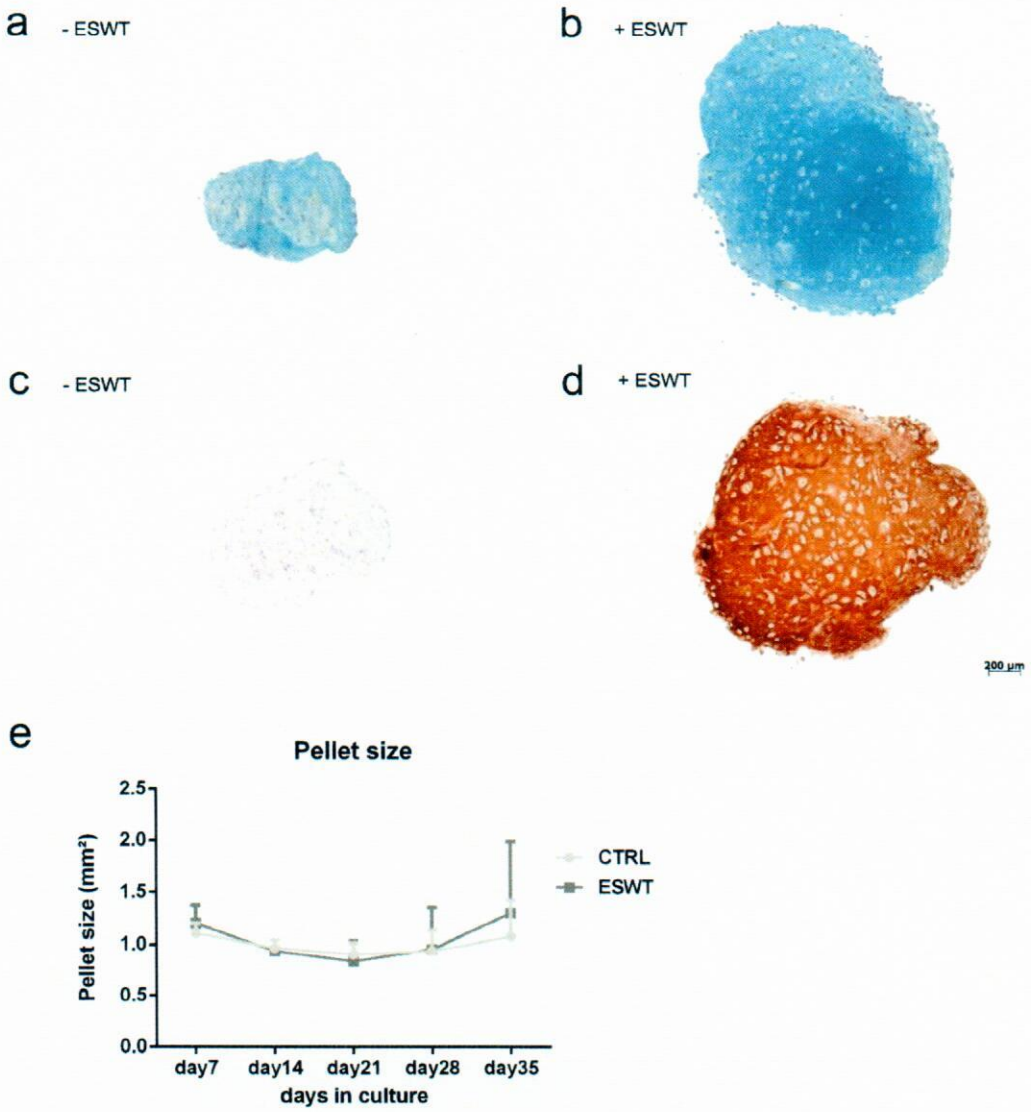

Figure 6. Extracorporeal shock wave therapy (ESWT) in situ increases the chondrogenic differentiation potential of isolated cells. ESWT treatment increased chondrogenic differentiation resulting in stronger staining of Alcian Blue (a, b) and collagen type II (c, d) after 35 days as well as a slightly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

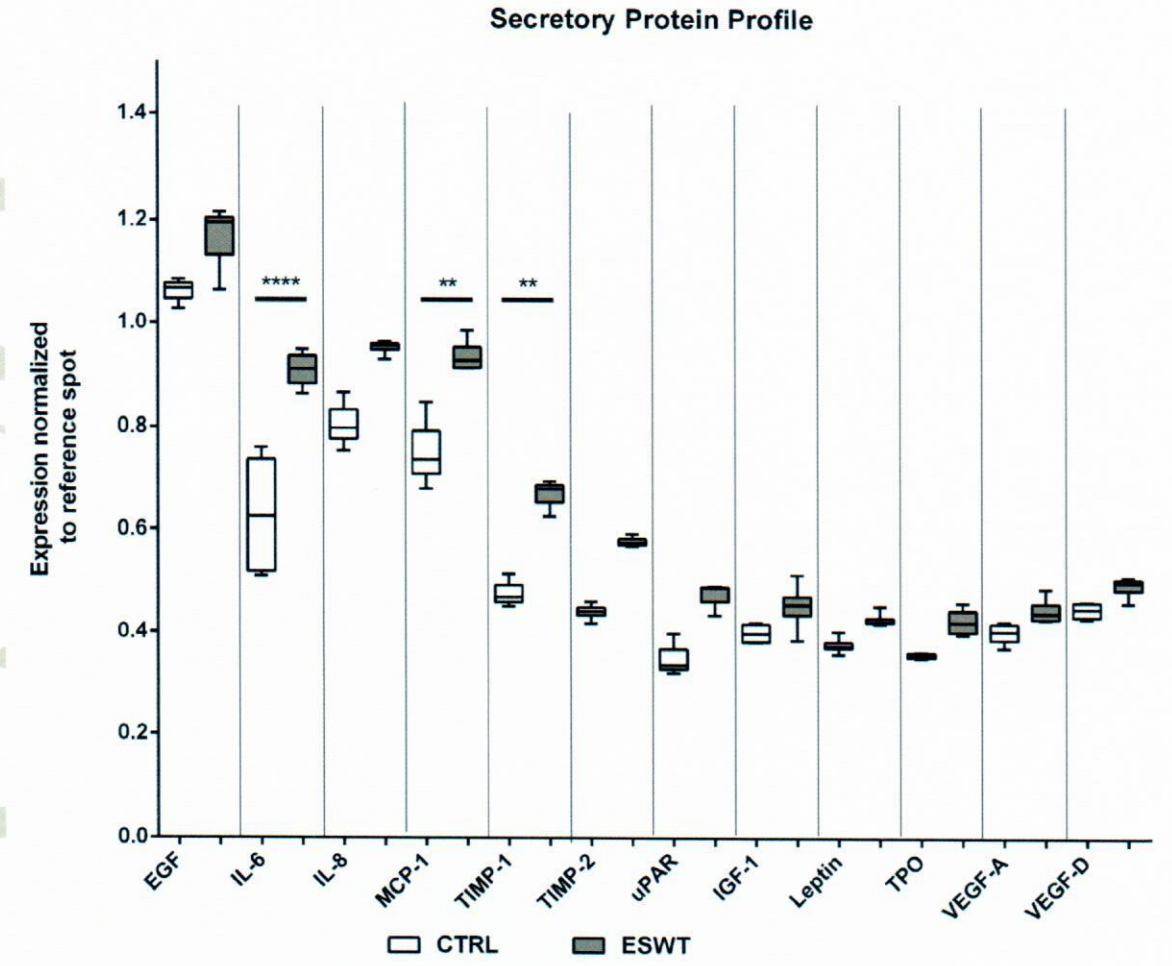

Figure 7. Extracorporeal shock wave therapy (ESWT) in situ changes the secreted angiogenic protein profile of isolated cells. ESWT treatment showed substantial changes in the expression of angiogenesis related proteins. Three analyzed proteins demonstrated a significantly enhanced expression after ESWT treatment: interleukin-6 (IL-6), monocyte chemotactic 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), interleukin-8 (IL-8), tissue inhibitor of metalloproteinase 2 (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 vascular endothelial growth factor D (VEGF-D). Data are presented as box-plots and represent duplicate measurements of 2 independent donors. **p < 0.01, ****p < 0.0001.