Improvement of adipose tissue–derived cells by low-energy extracorporeal shock wave therapy

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Abstract
Background. Cell-based therapies with autologous adipose tissue–derived cells have shown great potential in several clinical studies in the last decades. The majority of these studies have been using the stromal vascular fraction (SVF), a heterogeneous mixture of fibroblasts, lymphocytes, monocytes/macrophages, endothelial cells, endothelial progenitor cells, pericytes and adipose-derived stromal/stem cells (ASC) among others. Although possible clinical applications of autologous adipose tissue–derived cells are manifold, they are limited by insufficient uniformity in cell identity and regenerative potency. Methods. In our experimental set-up, low-energy extracorporeal shock wave therapy (ESWT) was performed on freshly obtained human adipose tissue and isolated adipose tissue SVF cells aiming to equalize and enhance stem cell properties and functionality. Results. After ESWT on adipose tissue we could achieve higher cellular adenosine triphosphate (ATP) levels compared with untreated control. Analysis of SVF protein secretome revealed a significant enhancement in insulin-like growth factor (IGF)-1 and placental growth factor (PLGF) after ESWT on adipose tissue. Discussion. Summarizing we could show that ESWT on adipose tissue enhanced the cellular ATP content and modified the expression of single mesenchymal and vascular marker, and thus potentially provides a more regenerative cell population. Because the effectiveness of autologous cell therapy is dependent on the therapeutic potency of the patient’s cells, this technology might raise the number of patients eligible for autologous cell transplantation.

Key Words: adipose tissue, adipose-derived stromal/stem cells, extracorporeal shock wave therapy, stromal vascular fraction

Introduction

Cell-based therapies with autologous adipose tissue–derived cells have shown great potential in several clinical studies in the last decades. In the field of aesthetic and reconstructive medicine an abundance of knowledge was accumulated in the last century [1] and later was extended through clinical studies in regenerative medicine and tissue engineering [2–4]. The majority of studies have been using the stromal vascular fraction (SVF), a heterogeneous mixture of fibroblasts, lymphocytes, monocytes/macrophages, endothelial cells, endothelial progenitor cells, pericytes and adipose-derived stromal/stem cells (ASC) among others [5–10]. In clinical case studies and trials treating soft tissue defects [4,11–14], bone and cartilage defects [15–19], gastrointestinal lesions [20], immune disorders [21,22], neurological injuries [23] and cardiovascular diseases [24], SVF and ASC have already proven their regenerative potential. Although possible clinical applications of autologous adipose tissue–derived cells are manifold, they are limited by drawbacks concerning stem cell identity and potency of the isolated cell population. Different cell isolation protocols and methods but also closed automated isolation devices bring up cell populations with...
variable content of potentially therapeutic cells within the fat graft or the SVF [25]. In addition, donor variability results in a highly heterogeneous cell composition and functionality, which may reduce reproducibility and efficacy, and increase the risk for transplantation of low-potent cells into the patient. SVF cells and ASC can be characterized with a distinct surface marker profile and have the ability to differentiate at least into the mesodermal lineages. This is defined in a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) together with the International Society for Cellular Therapy (ISCT) to provide guidance for standardization between different research groups [26]. Cultivation, purification and differentiation of ASC are standard procedures for clinical trials, but it remains difficult to meet the requirements of regulatory agencies for stem cell translation into clinics. To increase therapeutic cell potency, numerous strategies have been evaluated for activation of cells or cell material such as physical stimulation using low level light therapy (LLLT) [27–29], photobiostimulation [30,31] or radio electric asymmetric conveyer [32]. Moreover, the efficiency of ASC transplants was improved by the addition of activated platelet-rich plasma (PRP) [33,34] or growth factors [35].

In this study we aimed to improve stem cell properties and reduce donor variability by mild mechanical stimulation using extracorporeal shock wave therapy (ESWT).

Extracorporeal shock waves are sonic pulses, characterized by an initial increase, reaching a positive peak of up to 100 MPa within 10 ns, followed by a negative amplitude of up to -10 MPa and a total life cycle of less than 10 µs [36]. Biological responses are thought to be triggered by the high initial pressure, followed by a tensile force and the resulting mechanical stimulation [36]. ESWT has been applied for several decades in the clinics and has demonstrated beneficial effects on tissue regeneration in non-union fractures [37–39], ischemia-induced tissue necrosis [40] or post-traumatic necrosis, disturbed healing wounds, ulcers and burn wounds [41,42]. We have previously shown that low-energy ESWT enhances proliferation and differentiation of ASC lines in vitro [43,44]. These in vitro studies corroborate the clinical success of ESWT in wound healing, nerve regeneration and vascularization [45,46].

In our experimental set-up, low-energy ESWT was applied to freshly isolated SVF cells from human adipose tissue aiming to equalize and enhance cell properties and functionality. To limit the degree of manipulation of the cells during the SVF isolation process we applied in a second approach ESWT directly on the freshly obtained human adipose tissue and compared it with ESWT on isolated SVF cells. Based on this, we studied cellular adenosine triphosphate (ATP) content, immunophenotype, cell yield, viability, colony-forming unit fibroblast (CFU-F) assay and protein secretome of the SVF. Furthermore, we cultured ASC from these SVF and investigated proliferation and differentiation potential toward the adipo-, osteo- and chondrogenic lineage.

### Methods

#### SVF/ASC isolation

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 anesthesia. SVF isolation was performed as modified from Wolbank et al. [47] as follows. Briefly, 100 mL liposuction material was transferred to a blood bag (400 mL Macopharma) and washed with an equal volume of phosphate-buffered saline (PBS) to remove blood and tumescence solution. Afterward, for tissue digestion PBS was replaced with 0.2 U/mL collagenase NB4 (Serva) dissolved in 100 mL PBS containing Ca²⁺/Mg²⁺ and 25 mmol/L N-2-hydroxyethylpiperazine-NO-2-ethanesulfonic acid (HEPES; Sigma) and the blood bag was incubated at 37°C under moderate shaking (180 rpm) for 1 h. The digested tissue was transferred into four 50-mL tubes (Greiner). After centrifugation at 1200g for 7 min, the cell pellets were incubated with 100 mL erythrocyte lysis buffer (154 mmol/L ammonium chloride [Sigma], 10 mmol/L potassium bicarbonate [Sigma], 0.1 mmol/L ethylenediamine-tetraacetic acid [EDTA; Biochrom] in aqua dest) for 3 min at 37°C to eliminate red blood cells. The supernatant was aspirated after centrifugation for 5 min at 500g. The pellets were pooled, washed with PBS and filtered through a 100-µm cell strainer (Greiner). After another centrifugation step at 500g for 5 min, the supernatant was removed and the isolated SVF cells were cultured in endothelial growth medium (EGM-2; Lonza) at 37°C, 5% CO₂ and 95% air humidity or resuspended in EGM-2 for further analyses. To obtain the adherent cell fraction including ASC, SVF were seeded on a plastic surface in expansion media (EGM-2), and cultured to a subconfluent state. Media was changed every 3 to 4 days. Cells were detached with Accutase (PAA) for 5 min at 37°C and collected in a tube. After centrifugation the pellet was resuspended in EGM-2 and cells were quantified with trypan blue exclusion in a cell counter (TC-20; Biorad).

#### In vitro ESWT

For in vitro shock wave treatment, an unfocused electro-hydraulic device was used (Dermagold 100; MTS...
Medical) alone or in combination with a water bath to allow reproducible physical propagation and application of shock waves, as described by Holfeld et al. [48]. For shock wave treatment of adipose tissue, unfocused shock waves were applied after the first washing step of the lipoaspirate with the following parameters: 200 pulses at an energy level of 0.09 mJ/mm² with a frequency of 3 Hz on both sides of the blood bag (further referred to as “ESWT FAT”). Afterward, isolation of SVF was continued using enzymatic digestion as described above. For shock wave treatment of freshly isolated SVF cells, 1–3 × 10⁷ cells/mL suspended in a total volume of 1 mL were transferred into conical 15-mL polypropylene centrifuge tubes (PAA) and placed in front of the applicator inside a water bath. Unfocused shock waves were applied to SVF cells with the same parameters like for shock wave treatment of adipose tissue (further referred to as “ESWT SVF”). The principle of the experimental set-up is shown in Figure 1. Shock wave parameters were chosen according to previous studies [43] and preliminary experiments to maximize the effect of the ESWT while concurrently minimizing possible negative effects.

Cell yield and viability

Cell number and viability were determined using trypan blue exclusion and quantification in a cell counter (TC-20; Bio-Rad). For quantification of cell viability, the percentage of living cells compared with total cell count was analyzed with the cell counter.

Intra/extracellular ATP

The amount of intra/extracellular ATP is an indicator for the viability of the cells. To determine the intra/extracellular ATP concentration CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used and performed according to the manufacturer’s instructions. Freshly isolated SVF cells were seeded at a density of 1 × 10⁴ cells per well in a black 96-well plate (Greiner) in 100 µL EGM-2. After 2 h, 100 µL CellTiter-Glo Reagent were added to each well and the plate was gently agitated on a shaker for 2 min. Afterward the plate was incubated for 10 min in the dark. The combination of intracellular and extracellular ATP can be measured as CellTiter-Glo Reagent includes a cell lysis buffer. The luminescent signal was detected with a luminometer (GloMax; Promega) at an exposure time of 2000 ms and correlated to an ATP standard curve.

Proliferation

Proliferation potential was analyzed by determining the population doubling level (PDL). Freshly isolated SVF cells were seeded at a density of 5 × 10⁵ cells per T-25 culture flask and cultured in EGM-2. Media 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 further analysis of PDL, ASC were seeded at a density of 5 × 10^4 in T-25 culture flasks and cultured until passage 3. Cell number was determined after each passage.

**CFU-F assay**

A defined number of SVF cells (4, 20, 100, 500, 2500 and 12500) was seeded in each well of a 6-well plate (Sarstedt) and cells were cultured in EGM-2 for 14 days. Medium was changed on day 7 after cell seeding. After 14 days of culture, cells were fixed with 4% formaldehyde and stained with hematoxylin. Afterward, the cells were washed with tap water and stained with eosin solution (Roth/Lactan). The percentage of cells that formed visible colonies was calculated and compared with the total number of seeded cells.

**Flow cytometry analysis of cell surface marker**

Freshly isolated SVF cells were characterized using flow cytometry for cell surface marker characteristic for SVF. Cells were incubated with the following antibodies on ice and in the dark for 25 min: 2 µL anti-CD14 fluorescein isothiocyanate (FITC), 1.5 µL anti-CD34 FITC, 1.5 µL anti-CD45 phycoerythrin (PE), 1 µL anti-CD73 PE, 1 µL anti-CD90 PE (all mouse monoclonal; BD Bioscience), 1.2 µL anti-CD105 FITC (mouse monoclonal; Abcam), 2 µL anti-CD31 FITC (eBiosciences) and 2 µL CD146-PerCP (R&D). Cells were washed twice with 1.5 mL Cell Wash (BD) and centrifuged for 5 min at 250g. The supernatant was discarded and the cell pellet resuspended in 200 µL Cell Wash. Samples were analyzed on a BD FACS Canto II (BD Bioscience): 10,000 events were detected and flow cytometry data were evaluated with the use of FlowJo Version 8.8 (Tree Star Inc.). Specific subpopulations were analyzed from thawed SVF cells through the combination of antibodies against CD45, CD31, CD34 and CD146 as well as CD90 and CD146: endothelial progenitor cells (EPC; CD45−/CD31+/CD34+), pericyte-like cells (CD45−/CD31−/CD146+), supra-adventitial ASC (SA-ASC; CD45−/CD31−/CD146−/CD34+) and mesenchymal/pericytic subset (CD90+/CD146+). For staining, 5 × 10^5 cells in 50 µL PBS with 1% fetal calf serum (FCS) were incubated with 5 µL primary labeled antibodies at room temperature for 15 min in the dark. Cells were washed with 1.5 mL Cell Wash and centrifuged for 5 min at 400g. The supernatant was discarded and the cell pellet resuspended in 300 µL 1 × Cell Fix (BD; diluted 1:10 with aqua dest). Samples were stored at 4°C in the dark until analysis on a FACS Aria II (BD).

**Osteogenic differentiation and detection**

For osteogenic differentiation, cells 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, media was changed to osteogenic differentiation media Dulbecco’s Modified Eagle’s Medium (DMEM)-low glucose (Lonza) containing 10% FCS, 2 mmol/L L-glutamine (PAA), 100 U/mL penicillin/streptomycin (Pen/Strep) (Lonza), 10 mmol/L dexamethasone (Sigma), 150 µmol/L ascorbat-2-phosphate (Sigma), 10 mmol/L β-glycerophosphate (StemCell Technologies) and 10 nmol/L dihydroxy-vitamin D3 (Sigma) or control media consisting of DMEM:F12/L-glutamine (Lonza) with 10% FCS and 100 U/mL Pen/Strep. Media 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 h with 70% ethanol at −20°C and stained with alizarin red solution (Merck) for 15 min. 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 min. 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 (Tecan). The second method to analyze osteogenic differentiation is the detection of the activity of intracellular ALP, which is located on the surface of osteoblast cells and has been shown to be a biochemical indicator of bone maturation, mineralization and bone turnover. Cells were incubated with 100 µL PBS for 1 h at −20°C. Afterward, the cells were lysed for 1 h by addition of 100 µL PBS containing 0.5% TritonX-100 (Sigma). For quantitative detection of ALP activity, 100 µL substrate solution (4-nitrophenylphosphate; Sigma) 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 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 (Tecan). By creating a standard curve with known p-nitrophenol concentrations diluted in stop solution (0.5% TritonX-100 diluted in PBS 1:2) and measuring the corresponding absorption, the ALP activity of the samples was calculated.

**Adipogenic differentiation and detection**

For adipogenic differentiation, cells were seeded at a density of 1.4 × 10^4 cells per well in a 24-well plate in EGM-2 and incubated overnight. On the next day,
media was changed to adipogenic differentiation media DMEM-high glucose (Lonza) containing 10% FCS, 2 mmol/L L-glutamine, 100 U/mL Pen/Strep, 1 µmol/L dexamethasone, 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX; Sigma), 10 µg/mL insulin (Sigma) and 100 µmol/L indomethacin (Sigma) or control media consisting of DMEM:F12/L-glutamine with 10% FCS and 100 U/mL Pen/Strep. Media 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 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). Then the cells were washed with aqua dest, counterstained for 1–3 min with Mayer’s 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 (Tecan).

**Chondrogenic differentiation and detection**

For chondrogenic differentiation in 3-D micromass pellet cultures, 3 x 10⁵ cells were centrifuged in chondrogenic differentiation media (hMSC Chondro BulletKit; Lonza) containing 10 ng/mL bone morphogenetic protein (BMP)-6 (R&D) and 10 ng/mL transforming growth factor (TGF)-ß3 (Lonza) in screw cap microtubes. The tubes were placed in an incubator at 37°C, 5% CO₂ and 95% humidity with slightly open cap for gas exchange. After 2 days the pellets were transferred to 96-well U-bottom plates (Greiner) with fresh media. Media was changed every 2 to 3 days. After 35 days of differentiation, micromass pellets were fixed in 4% phosphate-buffered formalin overnight for histological analysis. The next day the pellets were washed in 1x PBS and dehydrated in increasing concentrations of ethanol. 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 Mayer’s hematoxylin. For collagen type II staining, sections were incubated with pepsin for 10 min at 37°C (AP-9007 RTU, Thermo Scientific). Endogenous peroxidase was quenched with freshly prepared 3% H₂O₂ for 10 min at room temperature, followed by normal horse serum 2.5% (Vector RTU) to block unspecific binding. Sections were incubated 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 HRP labelled Polymer; Dako) for 30 min and rinsed in TBS again. Bindings were visualized using Nova Red (SK4800 Vector Labs) for 6 min. Counterstaining was performed with Mayer’s hematoxylin for 2 min.

**Secretory protein profile**

For protein secretome analysis of freshly isolated cells, 5 x 10⁵ cells were seeded in a T-25 flask in 2.5 mL EGM-2. Cells were allowed to adhere for 2 h and subsequently, EGM-2 was changed to serum-free media (DMEM-low glucose with 2 mmol/L L-glutamine). To include also non-adherent suspension cells, cells were collected, centrifuged and returned to the flask. Then, 24 h after seeding the supernatant was collected and stored at −80°C until analysis.

For the analysis of the secretory profile a Human Growth Factor Array (RayBio) 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/sec). Subsequently, the biotinylated antibody was incubated overnight at 4°C, followed by another washing step. The membrane was incubated with horseradish peroxidase (HRP)-conjugated streptavidin for 2 h. Prior to detection, the membrane was washed with washing buffer. Signals were detected using enhanced chemiluminescence and recorded on an X-ray film. Signals were densitometrically quantified using ImageJ (National Institutes of Health).

**Statistical analysis**

All data in this study represent single values for each donor or mean ± standard deviation. Statistical analysis was performed using Prism6 (GraphPad Software, Inc.), paired t test (ATP analysis, flow cytometry of single marker), unpaired t test (cell yield, viability, CFU), two-way analysis of variance (ANOVA)-Tukey post hoc (flow cytometry of subpopulations, PDL, quantitative analyses of ALP, alizarin red, oil red O) or one-way ANOVA-Fisher’s least significant difference (LSD) post hoc (secretory profile). Statistical significance was considered for P < 0.05.

**Results**

**Comparison of ESWT on human adipose tissue to ESWT on SVF cells**

To identify the most beneficial set-up, ESWT was applied either directly on adipose tissue isolated via liposuction from five different donors or on freshly processed SVF cells derived from the same donors.
ESWT on adipose tissue enhances cellular ATP

Cellular ATP levels were assessed 2 h after cell seeding, comparing the untreated control group with ESWT-treated SVF and ESWT-treated adipose tissue. Cellular ATP concentration tended to decrease after ESWT on SVF cells (Figure 2a) to a mean of 181 ± 91 nmol/L compared with untreated cells (225 ± 119 nmol/L). Only one donor showed a slightly elevated ATP concentration after ESWT on SVF cells. In contrast, after ESWT on adipose tissue all donors showed a significantly (P = 0.0081) increased ATP concentration (Figure 2b) with a mean of 475 ± 204 nmol/L.

ESWT on SVF and adipose tissue positively affects cell surface marker expression

To determine the impact of ESWT on the cellular phenotype in the SVF, we investigated the surface marker expression of freshly isolated SVF cells. Treatment of ESWT on SVF cells led to an increased expression of mesenchymal stromal cell markers CD73, CD90 and CD105 in all donors compared with control (Figure 3; MSC marker) resulting in 40.4 ± 5.4% CD73-positive cells (versus 29.1 ± 5.4% for control), 67.3 ± 7.4% CD90-positive cells (versus 57.3 ± 9.0% for control) and 22.3 ± 3.8 CD105-positive cells (versus 12.2 ± 3.3% for control). Similarly, ESWT on adipose tissue also enhanced the percentage of MSC marker of all donors significantly (Figure 3; MSC marker). This increase was stronger compared with the effect of ESWT on SVF cells and resulted in 49.5 ± 4.7% CD73-positive cells, 76.0 ± 3.2% CD90-positive cells and 27.8 ± 6.9% CD105-positive cells. Furthermore, other marker profiles within the SVF were enhanced after ESWT. All donors showed an increase of the endothelial/pericytic marker CD146 after ESWT on SVF cells, which was significant after ESWT on adipose tissue (Figure 4; vascular marker). While in untreated SVF cells a mean of 16.7 ± 3.6% were CD146-positive, 21.4 ± 5.7% of ESWT-treated SVF and 28.2 ± 6.8% of cells derived from ESWT-treated adipose tissue were CD146-positive. Both forms of ESWT application increased the proportion of CD31 (endothelial marker) positive cells in all donors, which was more pronounced in cells derived from ESWT-treated adipose tissue (Figure 4; vascular marker). The mean CD31 expression of untreated cells was 25.8 ± 5.0%, whereas ESWT enhanced the expression to 32.2 ± 6.6% and 35.9 ± 4.5%. In contrast, expression of the hematopoietic progenitor/vascular endothelial marker CD34 was not enhanced in any donor after ESWT on SVF cells and in only 2 of 5 donors after ESWT on adipose tissue (Figure 4; vascular marker). Therefore, mean expression levels of CD34 were similar in all three conditions (45.5 ± 2.4% untreated, 43.4 ± 3.0% ESWT-treated SVF and 44.3 ± 3.1% ESWT-treated adipose tissue). There was a minimal percentage of the monocyte/macrophage marker CD14 present in all three conditions and no difference between the groups untreated, ESWT-treated SVF or ESWT-treated adipose tissue (Figure 5; hematopoietic marker; mean for control 6.3 ± 2.5%, ESWT SVF 6.4 ± 2.2% and ESWT FAT 6.8 ± 2.0%). Similarly, there was no difference in the expression of the hematopoietic marker CD45 after ESWT on SVF cells or ESWT on adipose tissue compared with control (Figure 5; hematopoietic marker) with mean values of 13.6 ± 2.6% for untreated cells, 12.9 ± 2.7% for ESWT-treated SVF cells and 12.3 ± 2.8% for SVF cells derived from ESWT-treated adipose tissue.

Because ESWT applied on adipose tissue revealed higher beneficial effects regarding ATP release and expression of stem cell/progenitor marker compared with ESWT applied on SVF, we decided to focus on this approach.
Specific subpopulations present after ESWT on adipose tissue

Freshly isolated SVF cells from untreated and ESWT-treated adipose tissue were examined for the presence of specific subpopulations. The percentage of EPC (CD45−/CD31+/CD34+) was similar with 8.1 ± 0.9% for cells from untreated and 10.1 ± 2.9% for cells from ESWT-treated adipose tissue (Figure 6a). There was a minor percentage of pericyte-like cells (CD45−/
CD31−/CD146+ for both conditions (1.7 ± 0.4% versus 1.5 ± 0.6%). The number of SA-ASC (CD45−/CD31−/CD146−/CD34+, 13.5 ± 4.2% versus 11.6 ± 3.4%) and of the CD90+/CD146+ mesenchymal/pericytic subset (48.0 ± 5.5% versus 50.0 ± 8.5%) was also not affected by ESWT treatment (Figure 6a). The total cell number of the EPC subpopulation (3.0 ± 0.9 × 10^6 versus 6.4 ± 2.6 × 10^6), the SA-ASC (5.0 ± 2.5 × 10^6 versus 7.9 ± 4.5 × 10^6) and the CD90+/CD146+ mesenchymal/pericytic subset

Figure 4. Immunophenotype of freshly isolated SVF: vascular marker. Freshly isolated ESWT-treated SVF cells and SVF cells derived from ESWT-treated adipose tissue were examined for cell surface marker expression and compared with untreated control. The hematopoietic progenitor/vascular endothelial marker CD34 was not affected after both ESWT treatments. The endothelial/pericytic marker CD146 and the endothelial marker CD31 were not affected after ESWT on SVF, but there was a significant increase after ESWT on adipose tissue. Data are shown as single values for each donor ± ESWT. Asterisks indicate significant difference between the groups of n = 5. *P < 0.05.
Figure 5. Immunophenotype of freshly isolated SVF: hematopoietic marker. Freshly isolated ESWT-treated SVF cells and SVF cells derived from ESWT-treated adipose tissue were examined for cell surface marker expression and compared with untreated control. There was a minimal percentage of cells positive for the monocyte/macrophage marker CD14 and the hematopoietic marker CD45 and no difference between the groups. Data are shown as single values for each donor ± ESWT. n = 5.

Figure 6. Subpopulations of freshly isolated SVF after ESWT on adipose tissue. SVF cell composition after ESWT on adipose tissue was analyzed for specific subpopulations. The percentage of EPC (CD45−/CD31+/CD34+), pericyte-like cells (CD45−/CD31−/CD146+), SA-ASC (CD45−/CD31−/CD146−/CD34+) and a mesenchymal/pericytic subset (CD90+/CD146+) was not affected after ESWT on adipose tissue (a). When analyzing the total cell number of subpopulations there was a minor but not significant increase of EPC, SA-ASC and the CD90+/CD146+ mesenchymal/pericytic subset. The number of pericyte-like cells was not changed (b). Data are shown as mean ± SD. n = 3.
(2.0 ± 0.1 × 10^7 versus 2.6 ± 0.3 × 10^7) was increased, however, not significantly, after ESWT. The number of pericyte-like cells (CD45−/CD31−/CD146+) was not changed after ESWT treatment (6.4 ± 10.1 × 10^5 versus 8.3 ± 1.7 × 10^5; Figure 6b).

Cell yield, viability and proliferation after ESWT on adipose tissue

After application of ESWT on adipose tissue, the isolated cell yield was slightly but not significantly increased from 3.3 ± 2.3 × 10^7 to 3.9 ± 2.9 × 10^7 cells (Figure 7a). Viability of the freshly isolated SVF cells was similar for untreated cells and cells derived from ESWT-treated adipose tissue with 80.1 ± 9.0% and 80.9 ± 7.6% living cells compared with total cell number (Figure 7b). Similarly, there was no effect of ESWT on the proliferation potential of cultured ASC derived from the SVF. Cells isolated from ESWT-treated adipose tissue showed similar PDL compared with untreated cells after 7 days (2.3 ± 0.7 versus 2.5 ± 0.8), 14 days (7.6 ± 1.4 versus 7.6 ± 1.2) and 21 days (15.7 ± 2.7 versus 15.4 ± 2.3; Figure 7c). Analysis of the colony-forming capacity of the cells revealed a slightly but not significantly enhanced frequency after ESWT treatment on adipose tissue from 4.1 ± 2.4% for control to 5.2 ± 2.9% for ESWT (Figure 7d). The effect of ESWT on SVF regarding cell yield, viability and proliferation is provided as a supplemental figure (Supplemental Figure S1).

Multilineage differentiation potential after ESWT on adipose tissue

ASC cultures derived from untreated and ESWT-treated adipose tissue and SVF were analyzed for their in vitro osteogenic, adipogenic and chondrogenic differentiation potential. Upon osteogenic induction, increase in ALP activity showed similar values for cells from untreated adipose tissue and cells derived from ESWT-treated adipose tissue (485 ± 201 µmol/L versus
485 ± 238 µmol/L; Figure 8a). Qualitative analysis of osteogenic differentiation with alizarin red staining showed similar mineralization and calcification for both conditions (Figure 8c and 8d). This observation was confirmed by quantitative analysis of alizarin red staining by showing an extinction of 1.4 ± 0.8 for untreated cells and 1.5 ± 0.7 after ESWT on adipose tissue (Figure 8b). Adipogenic differentiation analyzed by oil red O staining also showed a similarly high intensity after ESWT on adipose tissue (Figure 9b and 9c), which was verified by quantitative analysis (0.58 ± 0.15 versus 0.63 ± 0.14; Figure 9a). Figure 10 shows 3-D micromass pellets from three representative donors with high chondrogenic differentiation potential, demonstrated by strong alcian blue and collagen type II staining, but no difference was visible between control and ESWT treatment (Figure 10). The effects on the three lineage differentiation potentials after ESWT on SVF are provided as supplemental figures (Supplemental Figures S2–S4).

Secretory protein profile after ESWT on adipose tissue

To explore which molecular mechanisms are involved in the ESWT-induced changes supernatants of SVF cells were collected 24 h after isolation and protein expression was analyzed. All analyzed proteins (Figure 11 and Supplemental Figure S5) from the following families were expressed in the supernatant of SVF cells after ESWT treatment and without treatment (Figure 11a–11i): nerve growth factor (NGF), TGFβ, epidermal growth factor (EGF), colony-stimulating factor (CSF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF) family. There were only two of the 41 analyzed proteins significantly enhanced after ESWT treatment, otherwise only minimal changes in the protein expression between untreated and ESWT-treated supernatants were determined. Namely, the expression of placental growth factor (PLGF) was...

Figure 8. Osteogenic differentiation potential after ESWT on adipose tissue. Cells derived from ESWT-treated adipose tissue were analyzed for their osteogenic differentiation potential with ALP (a) and alizarin red staining (b–d) and compared with untreated control. The increase in ALP activity was similar for control and ESWT on adipose tissue n = 10 (a). Alizarin red staining demonstrated similar mineralization for control (c) and cells derived from ESWT-treated adipose tissue (d), which was confirmed by quantitative analysis n = 9 (b). Regarding quantitative analyses of differentiation, all conditions showed a significant enhancement of differentiated cells compared with undifferentiated cells (a, b). undiff, cells in control media without growth factors and stimuli; diff, cells in osteogenic differentiation media. Data are shown as mean ± SD. n = 9–10. Size bar = 100 µm.
enhanced from $0.20 \pm 0.08$ to $0.34 \pm 0.16$-fold after ESWT treatment (Figure 11g). The expression of IGF-1 was increased from $0.14 \pm 0.08$ to $0.32 \pm 0.11$-fold after ESWT treatment (Figure 11i).

**Discussion**

Based on our previous studies, application of low-energy ESWT is promising for promoting the regenerative quality of stem cells. Recently we have shown that shock wave treatment at an energy level of 0.09 mJ/mm$^2$ enhanced adipogenic, osteogenic and Schwann-like cell differentiation of human ASC [43]. We have shown that shock waves at 0.1 mJ/mm$^2$ and 300 impulses improve skin flap survival through neovascularization and early upregulation of angiogenesis-related growth factors in a rodent ischemic epigastric flap model [40]. In previous studies, we and others could observe enhanced VEGF expression after ESWT *in vitro* and *in vivo* (Holfeld *et al.* [49]; Peng *et al.* [50]; Mittermayr *et al.* [51]). Shock wave treatment further demonstrated enhanced proliferation by release of cellular ATP in the C3H10T1/2 mouse MSC line and human ASC in an energy-dependent manner. ATP release subsequently activates purinergic receptors and finally enhances proliferation via downstream Erk1/2 signaling [44]. This is supported by our current findings that ESWT increases cellular ATP concentration in the isolated SVF, which remained at higher levels throughout culture (of ASC) compared with untreated cells (data not shown). Viability of SVF cells was not negatively influenced by ESWT treatment. This is consistent with studies from Sun *et al.* [52] and Yu *et al.* [53] that describe that at our chosen shock wave settings (low energy, low pulses) the effect of ESWT on ATP concentration did not impact viability of the cells.

The efficacy of shock wave treatment depends on various parameters including shock wave settings, number and time points of treatment and used cell type or material. To meet the requirements of the regulatory agencies and to receive the classification “minimally manipulated” medicinal product, to be run under the legislation of tissue banks (e.g., in Europe) for the translation of cells and tissues into clinics, adipose tissue should not be further processed.

**Figure 9.** Adipogenic differentiation potential after ESWT on adipose tissue. Cells derived from ESWT-treated adipose tissue were analyzed for their adipogenic differentiation potential with oil red O staining and compared with untreated control. Oil red O staining demonstrated similar lipid droplet formation for cells derived from untreated (b) and ESWT-treated adipose tissue (c), which was confirmed using quantitative analysis (a). Regarding quantitative analysis of differentiation, all conditions showed a significant enhancement of differentiated cells compared with undifferentiated cells (a). undiff, cells in control media without growth factors and stimuli; diff, cells in adipogenic differentiation media. Data are shown as mean $\pm$ SD. n = 10. Size bar = 100 µm.
Therefore, we analyzed the effect of ESWT directly on adipose tissue for improvement of the cells within the graft. Furthermore, the treatment was performed in a blood bag, which is also compatible with a closed sterile isolation process and allows direct application of ESWT. The set-up described here avoids open processes and substantial manipulation and should hence be in accordance with GMP setting and potentially a one-step procedure within the operation theater.

To ensure highest possible cell quality for a successful transplantation we analyzed the isolated SVF cells immediately after isolation. We could demonstrate that ESWT applied on adipose tissue resulted in an activated cell population with higher cellular ATP content and higher percentage of a mesenchymal stem/stromal, endothelial and pericyte-like immunophenotype. Taking these cells into culture (as such defined as ASC) we could corroborate the MSC character that is defined by IFATS and ISCT [26] in assessment of the trilineage differentiation potential toward the adipo-, osteo- and chondrogenic lineage. Our improved isolation protocol generates cells with a high in vitro differentiation potential with and without ESWT.

Additionally, isolated SVF cells in suspension were treated with ESWT (previously described by Schuh et al. [43] for adherence selected propagated ASC). ESWT-treated cells showed enhanced stem cell marker profile, which is consistent with the findings of Schuh et al. However, ESWT applied on adipose tissue provides cells with higher ATP content compared with ESWT applied on the isolated SVF thereof. This may be explained by the presence of surrounding tissue during treatment, which may protect the cells from the reflected waves. Furthermore, the tissue microenvironment including ECM and cellular context might contribute to the beneficial effect of ESWT within a native tissue. Immunophenotype analysis revealed cell populations with an enhanced percentage of single mesenchymal and vascular marker, suggesting more therapeutically relevant cells after ESWT. Analysis of percentages of specific subpopulations in SVF showed no effect of ESWT on adipose tissue. The total cell number of EPC, SA-ASC and mesenchymal/pericytic subset was elevated, although not significantly. Differentiation potential toward the osteo-, adipo- and chondrogenic lineage of cells derived from shock wave-treated adipose tissue was as high as for untreated cells. In the present study we could not detect a substantial influence of ESWT treatment on the secretion of human growth factors from freshly isolated SVF. Only two of the analyzed proteins showed a significantly

Figure 10. Chondrogenic differentiation potential after ESWT on adipose tissue. Cells derived from ESWT-treated adipose tissue were analyzed for their chondrogenic differentiation potential with alcian blue and collagen type II staining and compared with untreated control. Alcian blue and collagen type II staining demonstrated similar intensity for control and cells derived from ESWT-treated adipose tissue. Images show three representative donors. n = 10. Size bar = 200 µm.
Figure 11. Secretory profile of freshly isolated SVF cells after ESWT on adipose tissue. ESWT treatment showed no substantial changes in the expression of all analyzed protein families (a–i). Only two of 41 analyzed proteins demonstrated a significantly enhanced expression after ESWT treatment: placental growth factor (PLGF) (g) and insulin-like growth factor-1 (IGF-1) (i). Data are shown as mean ± SD. n = 6. *P < 0.05.
enhanced expression after ESWT treatment: PLGF, belonging to the VEGF family, and IGF-1. This suggests that the positive effect of ESWT on adipose tissue might probably not be due to a substantial change in trophic factor secretion. Cells isolated from shock wave–treated adipose tissue exhibit a higher cellular ATP turnover compared with shock wave–treated SVF cells or untreated cells. Although shock wave treatment on cells in culture triggers an immediate ATP release and a subsequent proliferation as demonstrated by Weihl et al. [44], we did not observe increased proliferation of cell cultures derived from shock wave–treated tissue, which, however, represents a different experimental set-up. The clinical success of ESWT in wound healing has already been demonstrated [41,51]. This effect is probably triggered through ATP release [44]. Wang et al. showed that intracellular ATP delivery promotes wound healing in a diabetic rat model [58]. Howard et al. [59] observed in an animal model devoid of skin contraction reduced wound healing times and accelerated new tissue growth after delivery of ATP. Hence, a higher ATP content of the cell could potentially be beneficial for transplantation and integration of the cell material inside the surrounding tissue, which is still a major challenge. Shock wave–activated adipose tissue could be used as a source material for cells that meet the standard of cell therapeutics. This follows the concept of bringing autologous cells such as SVF back into the patient without further processing within a one-step procedure. Limitation of potential risk factors during cell processing is also the prerequisite for classification of a cell product as “minimally manipulated” according to the regulatory framework as defined by the European Medicines Agency (EMA) [60]. Ongoing SVF and ASC transplantation studies have so far shown heterogeneous results regarding stem cell potency and efficacy. This is dependent on many factors such as tissue processing [61,62], cell isolation [25,63,64], patients’ health [65,66], body mass index [45,67] or age [68-70]. We have shown that cells isolated from shock wave–treated adipose tissue responded to an external stimulus, leading to higher levels of ATP and enhanced expression of single mesenchymal and vascular marker. Hence, these cells have the potential to produce a higher amount of ATP, which could have a positive impact on the transplantation process even though the cell identity could not reach uniformity after ESWT. Because the effectiveness of autologous cell therapy is dependent on the potency of the patient’s own cells, this technology might raise the number of patients eligible for autologous cell transplantation.

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delivery of adenosine triphosphate enhanced healing process

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