Wet milling of large quantities of human excision adipose tissue for the isolation of stromal vascular fraction cells

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Abstract The isolation of stromal vascular fraction (SVF) cells from excised human adipose tissue, for clinical or research purposes, implies the tedious and time consuming process of manual mincing prior to enzymatic digestion. Since no efficient alternative technique to this current standard procedure has been proposed so far, the aim of this study was to test a milling procedure, using two simple, inexpensive and commercially available manual meat grinders, to process large amounts of adipose tissue. The

procedure was assessed on adipose tissue resections from seven human donors and compared to manual mincing with scalpels. The processed adipose tissues were digested and the resulting SVF cells compared in terms of number, clonogenicity and differentiation capacity. After 10 min of processing, either device tested yielded on average sixfold more processed material for subsequent cell isolation than manual mincing. The isolation yield of SVF cells (isolated cells per ml of adipose tissue), their viability, phenotype, clonogenicity and osteogenic/adipogenic differentiation capacity, tested by production of mineralized matrix and lipid vacuoles, respectively, were comparable. This new method is practical and inexpensive and represents an efficient alternative to the current standard for large scale adipose tissue resection processing. A device based on the milling principle could be embedded within a streamlined system for isolation and clinical use of SVF cells from adipose tissue excision.

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Introduction

Adipose tissue is an abundant, easily accessible source of a heterogeneous mixture of leukocytes, endothelial cells and a population of mesenchymal stromal cells (MSC), collectively named the stromal vascular fraction (SVF) (Gimble et al. 2011). The combination of all of these cell types has remarkable properties for autologous transplantation and tissue repair (Scherberich et al. 2013). Leukocytes and endothelial cells contribute to revascularization (Navarro et al. 2014), tissue engraftment (Scherberich et al. 2010) and the control of inflammation (Volat and Bouloumié 2013), whereas the MSC subpopulation has the ability to differentiate into adipose tissue (Ong and Sugii 2013), muscle (Choi et al. 2010) and cartilage or bone (Osinga et al. 2016). Clinically, autologous transplantation of adipose tissue is popular for breast reconstruction and augmentation (Largo et al. 2014), improvement of facial scar appearance and microcirculation (Pallua et al. 2014), facial volumetric restoration (Pasquale et al. 2015), vocal cord augmentation (Tamura et al. 2008), treatment of velopharyngeal insufficiency (Bishop et al. 2014), posttraumatic chronic ulcers (Klinger et al. 2010), cryptoglandular fistulae-in-ano (Borowski et al. 2015) or alleviation of neuropathic scar pain (Huang et al. 2015). The outcome of autologous transplantation has been demonstrated to be dependent on number and viability of SVF cells (Kølle et al. 2013) as well as differentiation potential of the enclosed MSC (Salem and Thiemermann 2010).

Recent clinical trials have exploited the availability of GMP-grade collagenase to directly extract large quantities of SVF cells from liposuction material and to create cell-based engineered grafts (Meijer et al. 2008; Saxer et al. 2016) for intraoperative use. However, in certain cases solid adipose tissue is obtained in large quantities instead of liquid liposuction material, making it highly desirable to develop protocols for the extraction of SVF cells from this source, otherwise unused and discarded, for therapeutic or research purposes. Major limitations of the current standard method for SVF extraction from solid adipose tissue include the slow, labor-intensive process and low yield. Typically, the tissue needs to be minced manually with two scalpel blades, and often less than 1 kg could be processed in a day. Although commercial tissue homogenizers for cell extraction

are available [GentleMACS Dissociator, Miltenyi Biotec (Emnett et al. 2016)], they are not designed to handle large volumes of dense connective tissue.

To address this issue, a novel method of excision fat processing, based on simple, inexpensive and commercially available kitchen devices, was introduced before enzymatic isolation of SVF cells. The aim was to demonstrate the feasibility and efficacy of SVF cell extraction after processing of the material with a manual meat grinder, without harming their phenotype and function. Adipose tissue was thus processed using two different grinder models or the manual standard, followed by enzymatic digestion. Isolated SVF cells were then compared in terms of number, viability, clonogenicity, phenotype and differentiation capacity following expansion.

Materials and methods

Tissue source, staining and processing

Abdominal excision fat was obtained from seven donors (2 male, 5 female, in average 49 years of age) following informed consent and approval of the local ethical committee (Ethikkommission beider Basel [EKBB], Ref. 78/07, extended in 2009). After skin removal, the tissue was divided into three portions of equal weight. One portion was minced into 4–5 mm pieces by an experienced operator using two scalpels (video 1). The other two portions were processed each with a commercially available manual meat grinder (video 2). Device 1 comprised of a stainless steel body of 145 mm length and 54 mm diameter and a plate with 5 mm pores (Fig. 1a, b). Device 2 was made of an aluminum body of 106 mm length and 45 mm diameter and a plate with 4.5 mm pores (Fig. 2a, b). Both devices had the same feeder and a 4-sided knife. Complete disassembly, cleaning of all surfaces and steam-based sterilization with a standard medical autoclave were readily possible. Vital whole mount staining of the adipose tissue both after manual and meat grinder aided processing was performed to visualize its components as reported previously (Nishimura et al. 2007). In brief, the tissue samples were incubated with the following reagents for 30 min: BODIPY 558/568 C12 to stain adipocytes, Alexa Fluor 488 Isolectin GS-IB4 conjugate to stain endothelial cells and Hoechst 33342 to stain all nuclei

Fig. 1 Device 1 meat grinder tested: Size 5 mincer (Westmark, Germany), in disassembled (a) and assembled state (b)

Fig. 2 Device 2 meat grinder tested: Compact meat mincer (Moha Swiss, Switzerland), in disassembled (a) and assembled state (b)

(all from Molecular Probes, Eugene, OR, USA). After washing, $33 \times 1273 \times 1273\text{-}\mu\text{m}$ three-dimensional samples were evaluated under a confocal microscope (Nikon A1R, Nikon Corp., Tokyo, Japan). The stromal vascular fraction (SVF) cells were distinguished from the adipocytes by localization of the nuclei: when the nucleus was found within a BODIPY-positive area, it was regarded as an adipocyte; when the nucleus was within or closely attached to a vessel, it was

considered to be a vasculature-associated cell e.g. endothelial cell, adipose derived stromal cell or mural cell, collectively named the SVF. If neither of these conditions applied, the cells were either fibroblasts or blood cells. To demonstrate the effectiveness of the two meat grinders compared to the manual mincing method, the processing method was timed per method and the weight of both in- and output material was measured every minute for 10 min processing adipose

tissue from one donor. While no tissue was discarded using the manual processing method, the difference between in- and output weight was defined as tissue loss. For demonstration purposes this was quantified processing adipose tissue from one donor using one device, representative for both. Both devices were repeatedly fully disassembled and steam sterilized in a standard autoclave (SteriClave 24 BHD, Cominox, Carate Brianza, Italy) according to the recommendations of the Center for Disease Control (CDC 2008) without noticeable deformation or abrasion by macroscopic inspection.

SVF isolation and culture

Minced tissue was incubated for 60 min at 37 °C in 0.15% (W/V) collagenase II (Worthington, Lakewood, NJ, USA) diluted in phosphate-buffered saline (PBS; Gibco, Life Technologies, Zug, Switzerland). SVF cells were collected by centrifugation followed by red blood cell lysis with ammonium chloride solution (Stemcell Technologies, Grenoble, France), resuspension in complete medium (CM: α -MEM supplemented with 10% fetal bovine serum, 1% HEPES, 1% sodium pyruvate and 1% penicillin–streptomycin–glutamine, all from Gibco) and filtering through a 70 μ m nylon mesh (BD Biosciences, Allschwil, Switzerland) as previously described (Güven et al. 2011). Nucleated cells were stained with Crystal Violet (Sigma-Aldrich Fluka Chemie AG, Buchs, Switzerland), counted using a Neubauer chamber and the viability assessed by staining with 0.4% Trypan Blue (Sigma). For assessment of colony forming unit fibroblasts (CFU-f), 500 cells per 78 cm² tissue culture plate were seeded, cultured for 14 days in CM with 5 ng/ml fibroblast growth factor-2 (R&D Systems, Abingdon, UK), resulting colonies were fixed with 4% paraformaldehyde in PBS, stained with Crystal Violet and counted as previously described (Güven et al. 2012).

Characterization by flow cytometry

The phenotype of SVF cells was determined by cytofluorimetric analysis. 1×10^5 SVF cells were incubated for 30 min at 4 °C with fluorochrome-conjugated antibodies to human CD31, CD34 and CD45 (all from BD Biosciences) and analyzed with a FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using FlowJo software v. 10.0.6

(Tree Star Inc., Ashland, OR, USA) and expressed as a percentage of positive cells over the total cell population.

Osteogenic differentiation

Osteogenic differentiation was performed by seeding 4×10^3 cells per well in a 96-well plate and culturing for 14 days in CM with 0.1 mM ascorbate-2-phosphate, 10 mM β -glycerophosphate and 10 nM dexamethasone (all from Sigma). Cultures with CM containing 5 ng/ml FGF-2 were used as controls. Matrix mineralization was assessed by quantification of hydroxyapatite deposition using the Osteoimage Mineralization Assay kit (Lonza, Basel, Switzerland) following the manufacturer's instructions. In brief, hydroxyapatite deposits were stained with the Osteoimage staining reagent, washed five times with the provided washing buffer and fluorescence was measured at 405 nm excitation and 520 nm emission using a Synergy H1 Hybrid Multi-Mode spectrofluorometer (Biotek, Luzern, Switzerland).

Adipogenic differentiation

Adipocytic differentiation was induced as described previously (Barbero et al. 2003). In brief, 1000 cells per cm² were seeded on tissue culture plates and kept in high glucose DMEM supplemented with 10% FBS, 1% HEPES, 1% sodium pyruvate and 1% PSG (all from Gibco) until confluent. The medium of control cultures was not supplemented, whereas adipogenic cultures were supplemented with 10 μ g/ml insulin, 1 μ M dexamethasone, 100 μ M indomethacin and 0.5 mM 3-isobutyl-1-methylxanthine for 72 h and 10 μ g/ml insulin only for the following 24 h. This cycle of supplementation was repeated three times. Cells were then fixed with 4% formaldehyde in PBS, stained with Oil Red O 0.3% (Sigma) and photographed with an Olympus IX50 microscope (Olympus Corporation, Shinjuku, Tokyo, Japan). Quantification was performed by dissolving the staining in isopropanol (Emsure, Merck Millipore, Billerica, MA, USA) and measuring the absorbance at 500 nm.

Statistical analysis

All results are expressed as mean values $\pm$ standard deviation. Data were analysed by two-way ANOVA

for donors and processing methods. Individual mean values were compared post hoc with Tukey's honest significance (HSD) test. The threshold for significance was set at $p < 0.05$. The analysis was performed with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Manual processing generated a total of 27 g of minced adipose tissue after 10 min. In comparison, both devices were approximately 10-fold faster, with device 1 reaching a total of 311 g and device 2 a total of 249 g (Fig. 3a). However, tissue processing rate with the two devices was not linear over time, due to necessary intermittent cleaning to free the devices from clogging by fibrous connective tissue. This fibrous tissue was discarded. Therefore, the efficiency of adipose tissue processing of both devices, defined as output material in percent of input, was approximately 50% and relatively stable over time (Fig. 3b). Even when considering this limitation, the devices still yielded six to sevenfold more adipose material per unit of time for subsequent digestion than manual processing which reached 100% efficiency. Vital whole mount staining of the processed adipose tissue samples revealed a partially disrupted capillary network around the adipocytes and a low number of lipid droplets (Fig. 4).

The average isolation yield with the manual method was $3.0 \pm 2.7 \times 10^5$ cells/ml of minced adipose tissue, whereas device 1 showed an average isolation yield of $3.2 \pm 2.2 \times 10^5$ cells/ml and device 2 an average of $3.8 \pm 3 \times 10^5$ cells/ml (Fig. 5a). Although the manual method resulted on average in 4.9×10^4 cells/ml less than the two devices, this difference was not significant. The interdonor variability in isolation yield of SVF cells observed here was high but consistent with previous reports (Osinga et al. 2015; Güven et al. 2011). SVF viability was high and not significantly different between the three methods of adipose mincing ($79 \pm 4\%$ for manual, $85 \pm 5\%$ for device 1 and $79 \pm 5\%$ for device 2, Fig. 5b). The distribution of subpopulations of SVF cells with the different milling methods was analyzed by cytofluorimetry for cellular expression of CD31, CD34 and CD45 (Fig. 6). This allowed comparison of the percentage of hematopoietic (CD45+), endothelial (CD 31+/34+) and stromal cells (CD31–/34+). An average of 32% hematopoietic cells ($40 \pm 16\%$ manual, $32 \pm 14\%$ device 1, $25 \pm 7\%$ device 2), 11% endothelial cells ($10 \pm 9\%$ manual, $12 \pm 11\%$ device 1, $10 \pm 9\%$ device 2) and 60% stromal cells ($58 \pm 21\%$ manual, $66 \pm 23\%$ device 1, $56 \pm 35\%$ device 2) were present, without significant differences between the experimental groups. An average of 15% clonogenic cells were found in SVF cells preparations, without significant differences between the mincing techniques ($13 \pm 7\%$ for manual, $15 \pm 10\%$ for device 1 and $16 \pm 14\%$ for device 2) (Fig. 7a).

Fig. 3 Comparison of tissue amount (measured in grams) processed (manually, device 1 and 2) per minute over a 10 min time period (**a**). Display of processing efficacy defined as output

given in percent of input. Due to the inability of fibrous tissue processing by both devices the efficacy was roughly 50%, as demonstrated with one of the two devices (**b**)

Fig. 4 Representative, three-dimensional confocal microscopic picture of vital adipose tissue after meat grinder processing and staining with immunofluorescence: BIODPY

for adipocytes (red), isolectin for endothelial cells (green), and Hoechst for all nuclei (blue). Scale bar = 200 μm . (Color figure online)

Fig. 5 Analysis of stromal vascular fraction from each processing method: Isolation yield (**a**) in cells per milliliter of processed adipose tissue (each donor represented by one symbol) and total cell viability (**b**). Values are mean $\pm$ SD

Osteogenic and adipogenic differentiation, respectively, assessed by quantification of mineralization and lipid accumulation by the expanded MSC, was not

significantly different between the mincing techniques (Fig. 7b, c).

Fig. 6 FACS profiles from cytofluorimetric analysis of the stromal vascular fraction (SVF) with fluorochrome-conjugated antibodies to human CD31, CD34 and CD45 for each of the three processing methods. Quantification showed no significant difference in distribution of subpopulations for the different processing methods. Values are mean $\pm$ SD

Fig. 7 Clonogenic potential (a) and multilineage differentiation potential of adipose-derived stromal cells (ASCs) from all three processing methods. Adipogenic (b) and osteogenic

(c) differentiation measured as fold of the differentiation capacity of cells from the manually processed tissue. Values are mean $\pm$ SD

Discussion

This study proposes an efficient method for the mincing of excision adipose tissue by using two inexpensive and easily available devices. We demonstrated that two different meat grinders were able to consistently produce more minced adipose tissue per unit time as compared to manual processing, without harming the SVF cells contained inside. Their use was validated by assessing SVF cell yield, viability, phenotype, as well as differentiation potential compared to the current standard method with manual processing.

Widely available kitchen devices were tested. The devices were easy to disassemble, clean and sterilize. No tissue residue was found upon cleaning and no abrasion could be found after sterilization. Considering the simple geometry of the devices in their disassembled form, no cellular, bacterial or viral contamination is expected upon thorough, appropriate cleaning and sterilization. However, dedicated testing of sterilization procedures should be performed before any clinical use.

Although one could assume that a meat grinder would exert mechanical stress during mincing, we did not find any difference in cell viability or functionality between manual- and meat grinder-based procedures.

Indeed, when comparing the average of 81% viability found here with adipose resections, with the published 70% of SVF viability after liposuction (Osinga et al. 2015), mincing seems less harmful to SVF cells than lipoaspiration. CFU-f frequency, a standard quality marker of SVF cells' growth under challenging low-density conditions, was reported as highly variable across studies (Fraser et al. 2008; Mitchell et al. 2006; Müller et al. 2010; Osinga et al. 2015), ranging from 0.2 to 32%. Our observations similarly point to a high interpatient variability of proliferative capacity, which is not influenced by any of the processing methods described here. Also the subpopulations present in the SVF cells were not altered by the processing methods. Although we used only key cell markers to define subpopulations, it is rather unlikely that sub-subpopulations would have been differentially affected by the mincing methods. However, future experiments could consider additional markers, e.g. to distinguish different endothelial progenitors/pericytes or specific subtypes of hematopoietic cells and leukocytes. A more in-depth analysis of subpopulations would be followed by functional analysis with dedicated assays for angiogenesis, inflammation and tissue organization.

We did not find a reduction in osteogenic or adipogenic differentiation capacity when using the meat grinder-based processing. Future studies should also analyze chondrogenic potentials, although currently there is a lack of standardized protocols for the assessment of chondrogenic potential by adipose derived MSC, which typically requires priming (Bianco 2014; Bianco and Robey 2015) by co-stimulation with BMP-6 (Hennig et al. 2007; Osinga et al. 2016).

In this study, manual mincing of adipose tissue typically yielded in $3 \pm 2.7 \times 10^5$ SVF cells per ml adipose tissue. The previously described number of SVF cells gained through liposuction material in automated systems was $3.0 \pm 1.0 \times 10^5$ cells/ml by CelutionTM from Cytosol Therapeutics (Lin et al. 2008) and $2.6 \pm 1.2 \times 10^5$ cells/ml through the Sepax[®] device from Biosafe SA (Güven et al. 2012) which is in line with published numbers after non-automated digestion of liposuction material of $3.0 \pm 1.4 \times 10^5$ SVF cells/ml (Mitchell et al. 2006). Adhering to strict isolation protocols (Mehrkens et al. 2012; Osinga et al. 2015, 2016), we observed a similarly high interpatient variability in the present study, yet this variability did not appear to be influenced by any of the processing

methods. The ratio of material processed by material inserted into the two devices was roughly 50%, implying a considerable amount of tissue loss. This is however acceptable regarding the seven times larger amount of tissue being processed with the new method described.

In conclusion, we have presented an alternative method to manual mincing for excision adipose tissue. As compared to the standard isolation method, the introduction of an inexpensive, easily available, detachable and sterilizable device enabled the processing of much larger quantities of adipose tissue, without affecting the number and functionality of cells retrieved following typical enzymatic digestion. Our results prompt for the development of a device with similar functionality as the prototype kitchen appliance but of clinical grade and possibly capable to be combined to a streamlined process with currently available digestion/SVF isolation systems. In such configuration, the described procedure may prove valuable for future clinical studies using SVF cells from excision fat.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the local ethical committee (Ethikkommission beider Basel [EKBB], Ref. 78/07 extended in 2009). All seven donors gave written informed consent.

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