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Comparison of intraoperative procedures for isolation of clinical grade stromal vascular fraction for regenerative purposes: a systematic review

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Running title

Intraoperative procedures for stromal vascular fraction isolation

Abstract

Introduction

Intraoperative application of stromal vascular fraction (SVF) of adipose tissue, requires a fast and efficient isolation procedure of adipose tissue. This review was performed to systematically assess and compare procedures currently used for the intraoperative isolation of cellular SVF (cSVF) and tissue SVF (tSVF) which still contains the extracellular matrix.

Methods

Pubmed, EMBASE and The Cochrane Central Register of controlled trials databases were searched for studies that compare procedures for intraoperative isolation of SVF (searched 28th of September, 2016). Outcomes of interest were cell yield, viability of cells, composition of SVF, duration, cost and procedure characteristics. Procedures were subdivided in procedures resulting in a cSVF or tSVF.

Results

Thirteen out of 3038 studies were included, evaluating eighteen intraoperative isolation procedures, were considered eligible. In general, cSVF and tSVF intraoperative isolation procedures had comparable cell yield, cell viability and SVF composition compared to a non-intraoperative (*i.e. culture lab-based collagenase protocol)* control group within the same studies. The majority of intraoperative isolation procedures are less time consuming than non-intraoperative control groups, however.

Conclusion

Intraoperative isolation procedures are less time-consuming than non-intraoperative control group with similar cell yield, viability of cells and composition of SVF and therefore more suitable for use in the clinic. Nevertheless, none of the intraoperative isolation procedures could be designated as preferred procedure to isolate SVF.

Keywords: Lipografting, Stromal vascular fraction, Adipose derived stem/stromal cells, Non-enzymatic isolation, Enzymatic isolation, Collagenase

1. Introduction

Adipose tissue seems to be an outstanding source for regenerative therapies, since it is an easy accessible source for adipose-derived stem or stromal cells (ASCs). Adipose tissue can easily be harvested with liposuction, a low risk procedure that can be performed under local anesthesia. Several clinical trials have been published using ASCs for soft tissue reconstruction (Tanikawa et al. 2013), cardiac repair (Perin et al. 2014), pulmonary repair (Tzouvelekis et al. 2013) and cartilage repair (Jo et al. 2014). All these trials show promising results for future use of ASCs in tissue repair and regeneration.

To harvest ASCs, adipose tissue or lipoaspirate is subjected to enzymatic dissociation followed by several centrifugation steps (Bourin et al. 2013), which is a relative long-lasting procedure that cannot be performed during surgery. The cell population obtained by this enzymatic digestion and centrifugation is the stromal vascular fraction (SVF), containing ASCs, endothelial cells, supra-adventitial cells, lymphocytes and pericytes (Eto et al. 2009, Bourin et al. 2013). ASCs *in vivo* are characterized as CD31min/CD45min/CD34pos/CD90pos/CD105low cells (Yoshimura et al. 2006). After isolation, the SVF can either be used directly in clinical procedures or can be cultured to increase the number of cells before using them in the clinic (Gir et al. , Suga et al. 2007). In case of cell culturing, only ASCs and their precursor cells (supra-adventitial cells and pericytes) are able to adhere and survive (Zuk et al. 2001, Zimmerlin et al. 2010). Upon passaging *in vitro*, the phenotype of ASCs starts to deviate from their *in vivo* phenotype (Spiekman et al., 2016): in this process CD34 surface expression is lost, while CD105 expression is up-regulated to mention a few (Yoshimura et al. 2006, Corselli et al. 2012). Alternatively,

administration of the enzymatically prepared vascular stromal fraction of adipose tissue might have a therapeutic capacity that is similar to cultured ASCs. Although, no formal scientific evidence exists, the consensus is, that the therapeutic benefit of SVF predominantly relies on the abundantly present ASCs.

The current protocol to isolate and culture ASCs from adipose tissue involves enzymatic digestion with collagenase. This is a laborious and time consuming protocol and requires a specialized culture lab (Good Manufacturing Practice facilities (cGMP)), which is not available in most peripheral hospitals (Gimble et al. 2010). Therefore, intraoperative procedures for SVF isolation are warranted, in particular systems that do not employ enzymatic treatment, such as mechanical dissociation.

At present, several (commercial) procedures are available for intraoperative isolation of SVF (Aronowitz et al. 2015, Oberbauer et al. 2015). These intraoperative isolation procedures differ in various aspects: isolation of a single cell SVF (cellular SVF (cSVF)) resulting in a pellet with hardly any volume or isolation of SVF cells containing intact cell-cell communications (tissue SVF (tSVF). Most of the enzymatic intraoperative isolation procedures result in a cSVF, because of the loss of cell-cell communications and extracellular matrix. In most of the non-enzymatic intraoperative isolation procedures the cell-cell communications remain intact, resulting in an end product with more volume (tSVF). Different studies assessed the cell yield and phenotype of the isolated cSVF or tSVF of the various intraoperative isolation procedures compared to other intraoperative (commercial) procedures or to the gold standard for SVF isolation (non-intraoperative culture lab-based collagenase protocols which require cGMP facilities for clinical use, referred to as 'non-intraoperative isolation protocol'). Recently, new intraoperative isolation procedures are introduced and tested. It is not clear yet if intraoperative isolation procedures generate a similar quality and quantity of SVF as non-intraoperative isolation protocols. Next to this, the distinction between end products of intraoperative isolation procedures, *e.g.* cSVF and tSVF have never been studied. Therefore, a systematic review was performed to assess the efficacy of intraoperative isolation procedures of human SVF based on number of cells, cell viability and composition of SVF. In addition, duration and costs of the intraoperative isolation procedures were compared.

2. Material & Methods

2.1. Protocol and registration

This study was performed using the PRISMA protocol (Moher et al. 2009). The search strategy for this systematic review was based on a Population, Intervention, Comparison, and Outcome (PICO) framework (Schardt et al. 2007). The study was not registered.

2.2. Eligibility criteria

Studies were included when at least two different types of intraoperative isolation procedures or one intraoperative isolation procedure with a non-intraoperative isolation protocol were assessed using human adipose tissue to isolate SVF. Studies need to use the adipose fraction of lipoaspirate. Studies only evaluating centrifugation forces, sonication or red blood cell (RBC) lysis buffer were excluded. Studies focusing on processing methods of adipose tissue for the use in fat grafting were excluded as well as case reports, case series and reviews. Searches were not limited to date, language or publication status (Table 1).

2.3. Information sources and search

Pubmed, EMBASE (OvidSP) and The Cochrane Central Register of controlled trials databases were searched (searched 28th September, 2016). The search was restricted to human studies. The search terms (Table 2) were based on three components: (P) adipose stromal cell, adipose stem cell, stromal vascular fraction, autologous progenitor cell, or regenerative cell in combination with (I) cell separation, isolation, dissociation, digestion, emulsification, isolation system, cell concentrator and finally connected with (C) enzymatic, non-enzymatic, or mechanical.

2.4. Study selection and data collection process

Two authors (JAD, AJT) selected studies independently based on the eligibility criteria. Inconsistencies were discussed during a consensus meeting. In case of disagreement, the senior author (MCH) gave a binding verdict.

2.5. Data items

Search term was partly based on a Population, Intervention, Comparison, Outcome (PICO) framework. Outcomes of interest were not included in the search term. For this review the outcomes of interest were cell yield, viability of the nucleated cells, composition of the SVF and duration, cost and characteristics of the intraoperative isolation procedures. Effect sizes were calculated on cell yield and viability in studies with a comparison of intraoperative isolation procedures versus regular non-intraoperative isolation protocols. Differences in harvesting procedure were not taken into account.

2.6. Risk of bias in individual studies

It is known that the quality of ASCs depends on age and harvest location of the donor (Engels et al. 2013, Dos-Anjos Vilaboa et al. 2014, Di Taranto et al. 2015, Maredziak et al. 2016). The inclusion of young healthy patients may positively affect the results. Therefore, detailed information about demographics are described in this review.

2.7. Summary measurements

Effect sizes were calculated of the outcome variables cell yield and percentage of viable nucleated cells from cSVF between enzymatic intraoperative isolation procedures and non-intraoperative isolation protocols (gold standard). The following effect size formula was used: effect size = (difference in mean outcomes between enzymatic intraoperative isolation procedures and gold standard) / (standard deviation of the gold standard). Studies which presented results in mean and standard deviation were analyzed. Intraoperative isolation procedures focusing on tSVF instead of cSVF were not taken into account in the effect size of cell yield, because of different start volumes of lipoaspirate and end volumes of tSVF.

2.8. Synthesis of results

In some studies, derivate numbers of graphs are used when the actual number of outcomes was not given. Cell types within the SVF can be distinguished based on CD marker expression or immuno-staining. To compare SVF compositions between different studies and to compare intraoperative procedures with their control (*i.e.* non-intraoperative protocols or other intraoperative procedures) in the same study, only CD marker expression was used. Studies evaluating a single CD marker expression to analyze different cell types were seen as insufficient distinctive and were excluded. Cells were divided into two major groups: CD45min (adipose tissue-

derived) and CD45pos (blood derived) cells to analyze the expression of stromal cells, pericytes, vascular endothelial cells/endothelial progenitor cells, endothelial cells, lymphocytes, leukocytes and hematopoietic stem cells. All other cells are placed in the category: other cell types. The CD34pos/CD146pos population is excluded from analysis because of the inability to discriminate between progenitor pericytes and progenitor endothelial cells (Bianchi et al. 2013).

2.9. Risk of bias across studies

Included studies could present different outcome variables related to SVF analysis. There is a risk that studies did not present a full SVF characterization and thereby bias their results. In order to provide an overview of the used outcome variables per study, a Modified IFATS/ISCT Index Score was used (see 2.10). The risk of publication bias of positive results might be expected in those articles were the authors have benefits in the investigated products. Disclosure agreements were reviewed for each study.

2.10. Modified IFATS/ISCT Index Score for the measurement of adipose tissue-derived stromal vascular fraction

Studies were assessed based on the reported outcome variables. The assessment of quality was evaluated based on the position statement of the International Federation of Adipose Therapeutics and Science (IFATS) and the International Society of Cellular Therapy (ISCT) (Bourin et al. 2013). The IFATS and ISCTS proposed guidelines to develop reproducible standardized endpoints and methods to characterize ASCs and SVF cells. For each of the following characterization methods a grade was given by the authors (JAD, AJT) to an article if the characterization was carried out: viability of nucleated cells, flow cytometry of SVF cells, flow cytometry of ASCs (CD13, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105, CD235a), proliferation and frequency (CFU-F) and functional assays (adipogenic, osteogenic and chondrogenic differentiation assays) of ASCs. The maximum score in case of a full characterization was 5.

3. Results

3.1. Included studies

A total of 3038 studies were identified after database searching. 2955 articles were excluded after abstract screening. 59 full text studies were assessed on eligibility criteria. Fourteen studies were excluded based on the use of a non-intraoperative protocol for isolation as experimental method (Yoshimura et al. 2006, Jiang et al. 2007, Pilgaard et al. 2008, Vykoukal et al. 2008, Al Battah et al. 2011, Fink et al. 2011, Condé-Green and Lamblet 2012, Okura et al. 2012, Carvalho et al. 2013, Escobedo-Lucea et al. 2013, Siciliano et al. 2013, Chen et al. 2014, Doi et al. 2014, Seaman et al. 2015). Seven studies described isolation protocols in general but gave no results (Hicok and Hedrick , Bernacki et al. 2008, Dubois et al. 2008, Yu et al. 2011, Zachar et al. 2011, Buehrer and Cheatham 2013, Zhu et al. 2013). Seven studies were excluded based on the lack of a control group (*i.e.* non-intraoperative isolation protocols or other intraoperative isolation procedures) (Zuk et al. 2001, Zeng et al. 2013, Dos-Anjos Vilaboa et al. 2014, Inoue et al. 2014, Sadighi et al. 2014, Van Pham et al. 2014, Raposio et al. 2016). Four studies were excluded based on their study design (Kim 2014, Marincola 2014, Aronowitz and Hakakian 2015, Bertheuil and Chaput 2015). Three studies were excluded based on the use of culture methods to isolate ASCs, because culture methods are incompatible with intraoperative applications (Wu et al. 2012, Busser et al. 2014, Priya et al. 2014). Four studies used only centrifugation, centrifugation or RBC lysis buffer as isolation protocol and were thereby excluded (Baptista et al. 2009, Markarian et al. 2014, Raposio et al. 2014, Amirkhani et al. 2016). Three studies used the blood saline fraction of lipoaspirate and were thereby excluded (Francis et al. 2010, Shah et al. 2013, Cicione et al. 2016). Four studies did not describe an outcome of interest (Reshak et al. 2013, Fraser et al. 2014, Yi et al. 2014, Aronowitz et al. 2015). Four additional studies were identified through other sources (Fig. 1). Thus, thirteen studies with eighteen intraoperative isolation procedures remained for analysis.

3.2. Study characteristics

In total, 93 subjects were enrolled in the thirteen studies. Nine studies reported gender of which 95% was female (n=58). Nine studies reported the mean age or age variance of the subjects and ten other studies described the use of infiltration (Table 1, supplemental content). No meta-analysis could be performed because the metrics and outcomes were too diverse.

3. 3. Characteristics of the intraoperative isolation procedures

All intraoperative isolation procedures are divided into two categories: enzymatic and non-enzymatic procedures resulting in cSVF and tSVF respectively (Table 3A and table 3B). Eight of the eighteen intraoperative isolation procedures were based on enzymatic digestion and ten isolation procedures were based on non-enzymatic procedures. Two non-enzymatic procedures, the Residual tissue of emulsified fat procedure and the Fractionation of adipose tissue procedure, are named differently, but are almost identical. One intraoperative isolation procedure, the Filtrated fluid of emulsified fat, is a combined procedure of two other intraoperative isolation procedures *i.e.* the Fractionation of adipose tissue procedure and the Nanofat procedure (Tonnard et al. 2013, Mashiko et al. 2016, van Dongen et al. 2016).

3.3.1 Start volume versus end product

The Automated isolation system, GID SVF2, Lipokit system and Multi station are enzymatic intraoperative isolation procedure that resulted in large average amounts of SVF (7.2 ml – 20 ml), suggesting inefficient enzymatic digestions (SundarRaj et al. 2015, Aronowitz et al. 2016). The non-enzymatic intraoperative isolation procedures resulted in larger end volumes than only a pellet. Prior the Lipogems procedure, 130 ml of adipose tissue can be obtained to mechanical dissociate to 100 ml of lipoaspirate. Hence, this a reduction of the volume of 1.3 times, suggesting an inefficient mechanical dissociation to our opinion (Bianchi et al. 2013). In contrast, the Fractionation of adipose tissue procedure resulted in a 10.4-fold volume reduction (van Dongen et al. 2016). For all other intraoperative isolation procedures, no data is mentioned about the end volume of the lipoaspirate (Table 3A and table 3B).

3.3.2 Duration and costs

Duration of the intraoperative isolation procedures varied from 5 seconds to 133 minutes (n=12). Isolation with the Automated isolation system was the longest intraoperative isolation procedure (SundarRaj et al. 2015). Shuffling lipoaspirate 5 or 30 times through a luer-to-luer lock syringe will take 5 or 30 seconds respectively and were therefore the fastest procedures (Osinga et al. 2015). In general, the tested non-enzymatic procedures take less time than the enzymatic procedures (Table 3A and table 3B).

The costs of only enzymatic procedures Celution system (2013: $1950 and 2016: $2400), CHA-station ($710), Multi station (2013: $460 and 2016: $250), Lipokit system (2013: $530 and 2016: $450) and GID SVF2 ($1000) are mentioned, the enzymatic Celution system being the most expensive (Aronowitz and Ellenhorn 2013, Aronowitz et al. 2016). No data of non-enzymatic intraoperative procedures were available (Table 3A and table 3B).

3.4. Cell yield

Thirteen studies evaluated the cell yield of eighteen different intraoperative isolation procedures (Millan , Lin et al. 2008, Guven et al. 2012, Aronowitz and Ellenhorn 2013, Bianchi et al. 2013, Doi et al. 2013, Tonnard et al. 2013, Domenis et al. 2015, Osinga et al. 2015, SundarRaj et al. 2015, Aronowitz et al. 2016, Mashiko et al. 2016, van Dongen et al. 2016) (Table 2A and table 2B, supplemental content). The reported cell yield after those different procedures varied between $0.19 - 11.7 \times 10^5$ cells per ml in enzymatic intraoperative isolation procedures and between $1.8 - 22.6 \times 10^5$ cells per ml in non-enzymatic intraoperative isolation procedures. Non-enzymatic intraoperative procedures yielded higher number of cells since the cell yield was based on 1ml of end volume, whereas the enzymatic intraoperative isolation cell yield was based on the obtained pellet per 1 ml start volume of lipoaspirate. Of the enzymatic intraoperative isolation procedures, the Celution system, Multi station and Lipokit system were evaluated by more than one group of authors (Lin et al. 2008, Aronowitz and Ellenhorn 2013, Domenis et al. 2015, Aronowitz et al. 2016). Interestingly, obvious different yields were seen using the same procedure in different studies (Lin et al. 2008, Aronowitz and Ellenhorn 2013, Domenis et al. 2015, Aronowitz et al. 2016). Reproducibility is thereby questioned in our opinion. The cell yield using the enzymatic Celution system was significantly higher as compared to the Lipokit system ($p=0.004$), the Multi station ($p=0.049$) and CHA-station ($p<0.001$) (Aronowitz and Ellenhorn 2013). In contrast, Domenis et al. did not find a statistical difference between the enzymatic Celution system and Lipokit system. Moreover, Aronowitz et al. again compared the enzymatic Celution system with the Lipokit system and Multi station. This time, Multi station and the Lipokit system resulted in significant more cells as compared to the Celution system ($p<0.05$) (Aronowitz et al. 2016).

In the non-enzymatic intraoperative isolation procedures, the Squeezed fat, Residual fluid of emulsified fat and Fractionation of fat procedures resulted in the relative highest cell yields per ml harvested lipoaspirate (Mashiko et al. 2016, van Dongen et al. 2016). Non-enzymatic intraoperative isolation procedures such as shuffling (5 times and 30 times), the Nanofat procedure and Fastem did not mention the begin and end volumes, so the relative yield by isolation cannot be calculated (Tonnard et al. 2013, Domenis et al. 2015, Osinga et al. 2015). Osinga et al, reported that most of the adipocytes remain intact after shuffling 5 or even 30 times (Osinga et al. 2015). Consequently, to our opinion, the effect of shuffling only cannot be stated as an isolation procedure. We deem it possible that the lipoaspirate after both two procedures did not differ from the initial lipoaspirate obtained at the start of the procedure. However, the benefit might be at a different level, because shuffling does improve the injectability of lipoaspirates as shown by Tonnard et al. (Tonnard et al. 2013).

More interesting than comparing intraoperative isolation procedures evaluated in different studies might be the comparison between an intraoperative isolation procedure and a non-intraoperative isolation protocol (gold standard) starting from the same lipoaspirate. Six studies reported the results of such comparisons (Table 4A) (Millan , Lin et al. 2008, Guven et al. 2012, Doi et al. 2013, Domenis et al. 2015, SundarRaj et al. 2015). The Automated isolation system and Tissue genesis cell isolation system resulted in the same cell yield as the non-intraoperative isolation protocol control (effect size, respectively, 0.07 and 0.00) (Doi et al. 2013, SundarRaj et al. 2015). Sepax isolated a higher cell yield compared to a non-intraoperative isolation protocol (effect size 1.11) (Table 4A) (Guven et al. 2012). Lower cell yield was seen after using the Lipokit system compared to the non-intraoperative isolation protocol control (effect size -0.52) (Domenis et al. 2015). Interestingly, the highest positive as well as the most negative effect sizes were seen with the enzymatic Celution system related to regular isolation with a non-intraoperative isolation protocol (Lin et al. 2008, Domenis et al. 2015).

3.5. Viability of nucleated cells

Eight studies described viabilities from 39% to 98% of nucleated cells in the SVF. No big differences in viability were seen between enzymatic and non-enzymatic intraoperative isolation procedures. The Filtrated fluid of emulsified fat procedure showed the lowest viability (Mashiko et al. 2016), while the Automated isolation system showed the highest viability of nucleated cells of 98% after isolation (Table 2A and table 2B, supplemental content) (SundarRaj et al. 2015). Three enzymatic and three non-enzymatic intraoperative isolation procedures were compared to a non-intraoperative isolation protocol regarding the viability of nucleated cells (Table 4B) (Lin et al. 2008, Doi et al. 2013, SundarRaj et al. 2015). The viability of five intraoperative isolation procedures was comparable to their non-intraoperative isolation protocol controls; the effect sizes were close to zero in many studies (Table 4B). Only the Filtrated fluid of emulsified fat procedure showed an effect size of -45.4 (Mashiko et al. 2016). In general, viability did not differ between non-intraoperative isolation protocols and the individual intraoperative isolation procedures tested.

3.6. Composition of stromal vascular fractions

The SVF compositions is reported in nine studies evaluating six enzymatic procedures and three non-enzymatic procedures. The stromal cell population is larger in the SVF isolated by the enzymatic Celution system, Sepax and Tissue genesis cell isolation system and the non-enzymatic Residual of emulsified fat and Squeezed fat procedures compared to other intraoperative isolation procedures (Guven et al. 2012, Aronowitz and Ellenhorn 2013, Doi et al. 2013, Mashiko et al. 2016) (Table 5, supplemental content). The percentage of stromal cell population of the SVF isolated by the enzymatic Celution system only differs with 25.2% between two studies (Aronowitz and Ellenhorn 2013, Domenis et al. 2015) and 32.8% between two other studies, both evaluated by Aronowitz et al. (Aronowitz and Ellenhorn 2013, Aronowitz et al. 2016). In general, non-enzymatic procedures yielded same amounts of CD31min/CD34pos stromal cells.

The stromal cell population, including pericytes, ASCs and supra-adventitial cells, are the most important cell types in regenerative therapies because of their paracrine effect and multi-lineage differentiation capacity (Zuk et al. 2001, Pawitan 2014).

Pericytes defined using other CD markers than to define the stromal cell population are placed separately in the table. The enzymatic Celution system evaluated by Lin et al. resulted in the lowest percentage of pericytes in the SVF (0.8%), but used more than three CD markers to detect pericytes (Lin et al. 2008). SundarRaj et al. resulted in a higher percentage (2.0%) of pericytes in SVF obtained by the Automated isolation system, but used only two CD markers to determine the pericyte population and other cell types (SundarRaj et al. 2015). The use of multiple CD markers results in a more specific population than the use of less CD markers and so a lower percentage of that specific cell type *e.g.* pericytes (Bianchi et al. 2013). Bianchi et al. used CD34min/CD146pos/CD90pos to detect the pericyte-like population in the SVF and isolated the highest percentage of pericytes using the non-enzymatic Lipogems procedure as compared to other intraoperative isolation procedures (Bianchi et al. 2013). However, Bianchi et al. mostly used other combinations of CD markers in comparison to other studies (Bianchi et al. 2013). This renders their SVF composition incomparable with SVF compositions obtained by other intraoperative isolation procedures.

The enzymatic procedures: Automated isolation system, Tissue genesis cell isolation system and Sepax isolated more endothelial progenitor cells in comparison to other intraoperative isolation procedures (Guven et al. 2012, Doi et al. 2013, SundarRaj et al. 2015). Nonetheless, more endothelial progenitor cells were not corresponding to less stromal cells or pericytes. In all differently obtained SVF, the origin of large numbers of cells remains unidentified. This is partly because not every study identified both adipose tissue-derived and blood-derived cell types, but probably not every subpopulation of all cell types is already known as well.

When donor variability is neutralized by the use of the same lipoaspirate, intraoperative isolation procedures resulted in different SVF compositions. Lipogems isolated significantly more pericytes and stromal cells than the non-intraoperative isolation protocol control (p<0.05) (Bianchi et al. 2013) (Fig. 2). The enzymatic Celution system resulted in significantly more endothelial progenitor cells in comparison with the CHA-system, Lipokit system and Multi station, which is not necessarily preferred (p=0.003) (Aronowitz and Ellenhorn 2013). All other intraoperative isolation procedures compared with non-intraoperative isolation protocols showed no significant differences.

3.7. Modified IFATS/ISCT Index Score for the measurement of adipose tissue-derived stromal vascular fraction

Modified IFATS/ISCT index scores ranged from 1 to 4.6 out of 5. Güven et al. scored 4.6 and presented the most complete characterization of the SVF and ASCs (Guven et al. 2012) (Table 5). Tonnard et al. scored 2 points, but had only used CD34 as a marker to identify a subpopulation in the SVF (Tonnard et al. 2013). Two studies used other methods than flow cytometry to determine the composition of SVF (Osinga et al. 2015, van Dongen et al. 2016). No studies were excluded based on a low number of outcomes of interest measured by the modified IFATS/ISCT Index Score, because five out of thirteen studies scored less than half of the possible points given. This high number of low scores given to studies underlines the need for standardization.

3.8 Disclosure agreements of included articles

A disclosure agreement of support by the manufacturer was provided in five of the thirteen studies (Lin et al. 2008, Guven et al. 2012, Aronowitz and Ellenhorn 2013, Bianchi et al. 2013, Doi et al. 2013) (Table 6, supplemental content). The company, which was mostly involved in the studies, was Cytori, the manufacturer of the enzymatic Celution system.

4. Discussion

Grafting of lipoaspirates and of SVF in particular, is a rapidly evolving treatment modality for scars and other skin defects, arthritis, neuropathy, diabetic ulcers to mention a few. Many of these, initially small scale, single center studies, are on the verge of expansion to multicenter placebo-controlled double-blind randomized clinical trials. An important prerequisite is the use of an efficient and standardized intraoperative isolation procedure of SVF. This systematic review shows that none of these procedures supersedes other procedures in terms of cell yield, viability and SVF composition while being time and cost efficient too when analyzed using the same lipoaspirate. However, three intraoperative isolation procedures (shuffling 5 times, shuffling 30 times and Lipogems) showed only a minimal reduction of the volume of lipoaspirate, implicating that most of the adipocytes still are intact. Consequently, these three procedures are methods of processing rather than isolation procedures (Bianchi et al. 2013, Osinga et al. 2015). Moreover, there is a wide variation in cell yield, viability of cells and composition of SVF when all

intraoperative isolation procedures are compared together. Study characteristics showed small and varied sample sizes regarding the number, sex and age of the donors. It is known that the cell yield and viability of SVF differ among donors, depending on age, harvest location and co-morbidities, such as obesity, of the donors (Engels et al. 2013, Dos-Anjos Vilaboa et al. 2014, Di Taranto et al. 2015, Maredziak et al. 2016, Pachon-Pena et al. 2016). This interdonor variability is a possible explanation for the variations found between several studies. To avoid variation bias, isolation procedures should be investigated using identical lipoaspirates in the same study. There are, however, differences between non-enzymatic and enzymatic isolated SVFs on a different level. Non-enzymatic isolation procedures resulted in larger volumes (tSVF) than the resulting pellets (cSVF) after enzymatic intraoperative isolation procedures. Because the final products of both types of isolation procedures are different, the clinical purpose of the use of SVF is an important factor which isolation procedure suits best. In some cases, such as the intra-articular injection of SVF in temporomandibular joints requires very small volumes, whereas the end volume of SVF enriched lipofilling is less relevant. Isolation procedures of SVF of adipose tissue are based on reduction of large volume containing tissue or cells, such as ECM and/or adipocytes to concentrate the stromal vascular fraction. Non-enzymatic isolation of SVF results in a smaller volume of adipose tissue containing intact ECM and cell-cell communications between SVF cells (tSVF), because the shear forces are too low to disrupt cell to cell and cell to ECM adhesions (Lin et al. 2008, Corselli et al. 2012). Therefore, the tissue structure of lipoaspirate is still intact in the tSVF. Enzymatic procedures, however, likely result in a single cell cSVF, because enzymes likely disrupt all cell-cell interactions and ECM (Fig. 3) (Aronowitz et al. 2015). This is may not happen in the Automated isolation system, GID SVF2, Lipokit system and Multi station, possibly due to insufficient enzymatic digestion (SundarRaj et al. 2015, Aronowitz et al. 2016).

Clinical use of tSVF has several advantages over the use of cSVF in different clinical applications of regenerative medicine. It is well known that single cells migrate within 24 hours after application (Parvizi and Harmsen 2015). The ECM, containing a microvasculature structure, might function as a natural scaffold for cells like ASCs and most likely also augments rapid vascularization and reperfusion. This will probably increase cell retention rates after injection and enhance clinical effects. In case of early scar formation, wound healing, or organ fibrosis, tSVF might

therefore be more an appropriate therapy, which implicates that non-enzymatic procedures are more suitable as compared to enzymatic isolation procedures. In case of excessive pre-existing scar formation, the ECM in the SVF might not be appropriate and therefore the application of a cSVF or ASCs might be more eligible. ASCs could remodel excessive scar formation by immunomodulation or instruction of resident cells.

Characterization of subpopulations in the SVF depends upon selection of appropriate markers. Selection of an insufficient number of markers will give a disfigured image of the actual SVF composition (Fig. 3). SVF of adipose tissue can be divided into two major subpopulations based on the expression of CD45, which is a hematopoietic cell marker: adipose derived (CD45min) and blood derived (CD45pos) (Yoshimura et al. 2006). Adipose derived cell populations can be divided into endothelial cells (CD31pos) and stromal cells (CD31min) (Yoshimura et al. 2006). Three important subpopulations of the stromal cell population (CD45min/CD31min) are supra-adventitial cells: CD34pos/CD146min, pericytes: CD34pos/min/CD146pos and ASCs: CD34pos/CD90pos/CD105low (Yoshimura et al. 2006, Zimmerlin et al. 2010, Corselli et al. 2012, Corselli et al. 2013). Supra-adventitial cells and pericytes are both identified as precursor cells of ASCs, although there remains some controversy about this item (Lin et al. 2008, Traktuev et al. 2008, Zimmerlin et al. 2010, Corselli et al. 2012). Ideally, to discriminate between those three cell types within the CD45min/CD31min subpopulation, CD146 and/or CD90 markers should be used additionally. However, in most studies two CD markers or inappropriate combinations of CD markers have been used to determine cell types; only Lin et al. used all the aforementioned combinations (Lin et al. 2008). Because Lin et al. focus mainly on blood derived cells and not on the stromal cell population or pericytes, this did not affect their results. Doi et al. ascribed CD31min/CD34min/CD45min to the pericyte population, so therefore the CD34pos pericytes will be missed (Doi et al. 2013). SundarRaj et al. and Güven et al. used CD34pos/CD31min to determine the number of ASCs (Guven et al. 2012, SundarRaj et al. 2015), while pericytes and supra-adventitial cells also express CD34. Therefore, the number of ASCs contains pericytes and supra-adventitial cells as well (Yoshimura et al. 2006, Zimmerlin et al. 2010). To cover pericytes, supra-adventitial cells and ASCs, Domenis et al., Aronowitz et al. and Mashiko et al. used CD34pos/CD31min/CD45min to determine the stromal cell population (Aronowitz

and Ellenhorn 2013, Domenis et al. 2015, Aronowitz et al. 2016, Mashiko et al. 2016). CD34pos is frequently used as a marker to describe cells with stem cell characteristics in both hematopoietic and non-hematopoietic stem cells (Suga et al. 2009). The differences in use of CD marker expression to determine pericytes and the stromal cell population might be a possible explanation for the large variations found in SVF between different studies. No solid conclusions could be made about which isolation procedure generates the most stromal cells or pericytes.

Unfortunately, a limited number of commercially available intraoperative SVF isolation procedures not yet have reached scientific validation at an acceptable level. The American Society for Aesthetic Plastic Surgery (ASAPS) and the American Society of Plastic Surgeons (ASPS) published a position statement in 2012 on fat grafting and stem cells (Eaves et al. 2012). All specialized equipment for the use of stem cell extraction should be fully verified regarding efficacy and safety before use in clinical settings. In 2013, the IFATS and ICTS proposed guidelines with standardized endpoints and methods to verify and compare SVF isolation procedures (Bourin et al. 2013). None of the included studies fully verified their isolation procedure according to these IFATS and ICTS guidelines. Moreover, viability was measured in different ways among studies (*e.g.* directly on obtained SVF or after an extra non-intraoperative isolation protocol) and lipoaspirate was processed differently prior to isolation (*e.g.* centrifugation or decantation). For those reasons, we propose new adjusted IFATS and ICTS guidelines to validate intraoperative isolation procedures (Fig. 3). All intraoperative isolation procedures should be validated using centrifuged adipose tissue to determine the actual volume of lipoaspirate prior to isolation. It is known that increased centrifugal forces have a harmful effect on the viability of fat grafts (Xie et al. 2010, Tuin et al. 2016). However, the use of centrifuged adipose tissue is necessary to determine the actual cell yield after an isolation procedure. Furthermore, cell viability of tSVF should be determined directly on tSVF, instead of using an extra non-intraoperative isolation protocol which possibly results in more cell damage. However, the proposed adjusted standardized endpoints and methods by IFATS and ICTS are time-consuming and expensive since it requires cultured ASCs. In order to quickly verify isolation procedures intraoperatively during clinical trials, the end product of non-enzymatic intraoperative isolation procedures should be centrifuged to separate the oily fraction from the tSVF and pellet fraction based on density. For enzymatic intraoperative isolation

procedures, microscopy can be used to visualize single cells. In this way, isolation procedures can be quickly evaluated during clinical trials.

A large number of SVF isolation procedures without applying a full verification according to the IFATS and ICTS guidelines is available (Oberbauer et al. 2015). Oberbauer et al. presented a narrative overview of enzymatic and non-enzymatic intraoperative SVF isolation procedures (Oberbauer et al. 2015). In twenty-one out of thirty (both enzymatic as well as non-enzymatic) intraoperative isolation procedures reported in their study, there was a lack of verification data. In two studies intraoperative isolation procedures without scientific evidence *e.g.* viability of SVF, flow cytometry of SVF cells and ASCs, were used to treat patients. One study used SVF obtained by ultrasonic cavitation to treat patients with migraine and tension headache (Bright et al. 2014). Another study used SVF in combination with platelet rich plasma for meniscus repair (Pak et al. 2014). Hence, it cannot be guaranteed that the isolation procedures indeed isolate SVF, which is clinical safe for use. It seems that the use of most SVF isolation procedures with its concomitant clinical application is far ahead of a sound scientific base upon which these procedures should be used.

Moreover, the clinical safety of isolated SVF or ASCs is not clear yet, especially regarding clinical use in patients with any kind of malignancy. It is demonstrated, *in vitro*, that ASCs influence growth, progression and metastasis of cancer cell lines through *e.g.* promoting angiogenesis and differentiation of ASCs into carcinoma-associated fibroblasts (Freese et al. 2015). Zimmerlin et al. showed *in vitro* that ASCs influence growth of active malign cell lines, but this is not seen in latent cancer cell lines (Zimmerlin et al. 2011). Clinical data suggest that the use of isolated SVF or ASCs is safe in patients without an oncological history (Charvet et al. 2015). *In vitro* studies often use higher concentrations of ASCs as compared to clinical studies and this might be the cause of differences found between *in vitro* and *in vivo* studies (Charvet et al. 2015). However, to test clinical safety it is important to reach scientific validation of the commercially available procedures at an acceptable level. In this review it become clear that the reproducibility of the procedures as well as characterization of the SVF had shortcomings. If this is reached, further scientific research with proper controls with regard to the clinical effect and safety of SVF or ASCs are definitely wanted.

5. Conclusion

There is no evidence thus far that any intraoperative isolation procedure could be designated as preferred procedure for isolating SVF. However, three isolation procedures are rather processing techniques than isolation procedures. Enzymatic and non-enzymatic procedures had comparable results as it comes to cell yield, viability, and SVF composition. Non-enzymatic isolation procedures end products resulted had greater volumes (tSVF) than the pellets (cSVF) of the enzymatic isolation procedures. The results of intraoperative isolation procedures are comparable with those of the gold standard, the collagenase based non-intraoperative isolation protocol. Since intraoperative isolation procedures are less time-consuming, but as efficient as the non-intraoperative isolation protocol, the use of intraoperative isolation procedures seems to be more suitable for clinical purposes. However, only small sample sizes have been used to validate the isolation procedures. To test clinical safety, it is important to reach scientific validation of the commercially available procedures at an acceptable level. Regarding to this review, this level is not yet reached by many procedures.

6. Acknowledgement

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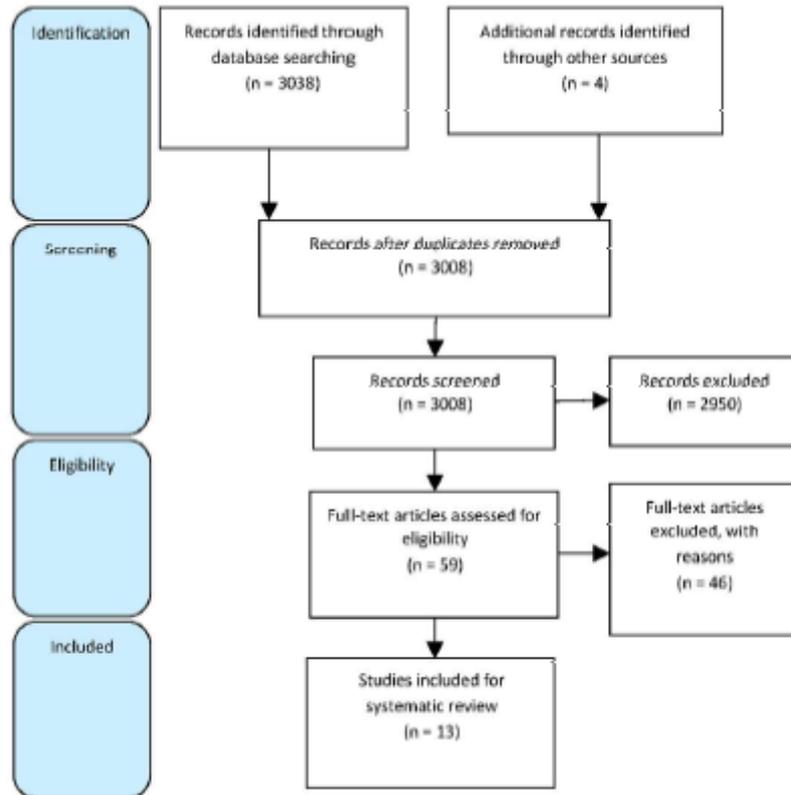
Figure 1.

Figure 1. Flow diagram of study selection

Figure 2.

Figure 2. SVF composition (CD marker) of procedures comparing an intraoperative isolation procedure with a non-intraoperative isolation protocol or with other intraoperative isolation procedures within one study. Stromal cell population (CD31min/CD34pos) consists of supra-adventitial cells, ASCs and pericytes, only pericytes defined as CD31min/CD146pos, CD31min/CD34min/pos or CD34min/CD146pos/CD90pos are placed separately in the table. Endothelial cells and vascular/progenitor endothelial cells are described as respectively, CD31pos/CD34min and CD31pos/CD34pos. No exact data described in text by Aronowitz et al., Bianchi et al., Domenis et al., Güven et al. and Mashiko et al., data is extracted from figures by authors JAD and AJT. AIS Automated Isolation System; CHA-station (CHA-Biotech); CYT Celution System Enzymatic (Cytori); FAST Fastem Corios (Corios); GID SVF2 (GID Europe); LIPOK Lipokit System (Medi-khan); PNC Multi station (PNC); REF Residual tissue of emulsified fat; SEPAX Sepax (Biosafe); SF Squeezed fat;Tissue Genesis Cell Isolation System (Tissue Genesis)

Figure 3. A) Schematic overview of enzymatic versus non-enzymatic intraoperative isolation and characterization of the obtained cSVF or tSVF. **B)** Legend of figure 3A.

Table 1. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Clinical trials	Case reports
Comparative studies	Case series
Full text available	Reviews
All languages	Letters to editor
Human studies	Non-comparative studies
	No full text available
≥2 different types of SVF isolation procedures	Processing methods for fat grafting
	Protocols using centrifugation or RBC lysis buffer only
1 SVF isolation procedure compared with control group	Mesenchymal cells derived from other source than adipose tissue
	Blood saline fraction used instead of adipose fraction of the lipoaspirate
Intraoperative procedures	Laboratory based enzyme protocols as experimental group
	No outcome of interest: SVF composition (CD markers), cell yield, viability of SVF

Table 2. Specific search terms of databases

Search term Pubmed:
((((Adipose Tissue [Mesh] OR Adipocytes [Mesh] OR Fat [tiab] OR Lipoaspirate* [tiab])) AND (Cell separation [Mesh] OR Isolat* [tiab] OR Dissociat* [tiab] OR Emulsification [tiab] OR Concentrat* [tiab] OR Digest* [tiab] OR Obtained [tiab])) AND (Stem cells [Mesh] OR Stromal cells [Mesh] OR Autologous progenitor cell* [tiab] OR Stromal vascular* [tiab] OR Regenerative cell* [tiab] OR Vascular stroma [tiab]))   *Restriction: Only human*
Search term Embase:
('adipose tissue':ab,ti OR 'adipocytes':ab,ti OR 'fat':ab,ti OR lipoaspirate*:ab,ti AND ('cell separation' OR isolat*:ab,ti OR dissociat*:ab,ti OR 'emulsification':ab,ti OR concentrat*:ab,ti OR digest*:ab,ti OR 'obtained':ab,ti) AND ('stem cells':ab,ti OR 'stromal cells':ab,ti OR 'autologous progenitor cell':ab,ti OR 'autologous progenitor cells':ab,ti OR 'stromal vascular':ab,ti OR 'stromal vascular fraction':ab,ti OR 'regenerative cell':ab,ti OR 'regenerative cells':ab,ti OR 'vascular stroma':ab,ti)) AND [embase]/lim NOT [medline]/lim AND 'article'/it   *Restriction: Only EMBASE*
Search term Cochrane Library:
(adipose tissue OR adipocytes OR fat OR lipoaspirate*) AND (cell separation OR Isolat* OR Dissociat* OR Emulsification OR Concentrat* OR Digest* OR Obtained) AND (stem cells OR stromal cells OR autologous progenitor cell* OR stromal vascular* OR regenerative cell* OR vascular stroma)

Table 3A. Duration, costs and procedure characteristics of intraoperative isolation procedures focusing on cSVF

Name	Author	Enzymatic/ Non-enzymatic (E/N)	Automatic/ Manual/ Semi (A/M/S)	Open/ closed (O/C)	Isolation details		Time (min)	Disposable (D)/ reusable (R) cost (Dollar)	Volume processed (ml)	Capacity (ml)	End volume (ml)	Maximum volume processed /maximum end volume
AIS	SundarRaj et al. 2015	E	A	C	Tissue digestion, heating and agitation, three-stage filter system (100 micron, 35 micron, 5 micron porosity)	-	133	-	-	500	10.8 [4-20]	-
CHA	Aronowitz et al. 2013	E	S	C	Fat bag, adapter, centrifugation, shaking incubator and tissue digestion, cell strainer, cell counter	Collagenase	88+/23	D710	80-180	180	-	-
	Aronowitz et al. 2013						90 +/16	D1950	100-180	360	-	-
CYT	Aronowitz et al. 2016	E	A	C	Washing (lactated Ringer), tissue digestion and agitation, centrifugation	Celase/ Reagent A	89.4 [85-93]	D2400 per 120-360 ml	126 [90-150]	360	5 [5]	30
	Domenis et al. 2015						60	D	250	-	Pellet	-
	Lin et al. 2008						90	-	-	-	Pellet	-
GID-SVF2	Aronowitz et al. 2016	E	M	C	Disposable canister for harvesting, filtration, separation and concentration	GIDzyme-50	71.4 [68-75]	D1000 per 20-120 ml	53.2 [32-88]	120	7.2 [6-9]	13.3
	Domenis et al. 2015					Liberase (collagenase mixture)	-	-	-	-	-	-
LIPOK	Aronowitz et al. 2013	E	S	C	1200 xg centrifugation (with a weight-mesh filter piston), celltibator	Collagenase	111+/-18	D530	60-100	100	-	-
	Aronowitz et al. 2016					Time Machine accelerator	120.8 [99-149]	D450 per 100 ml	71.4 [40–97]	400	20 [15-25]	3.9
PNC	Aronowitz et al. 2013	E	M	O	Centrifugation, shaking incubator, clean bench, HEPA filter, UV-lamp	Collagenase	115+/-13	D460	100-150	400	-	-
	Aronowitz et al. 2016						65.4 [59-74]	D250 per 100 ml	105.6 [68-150]	800	12.2 [10.5-15]	10
SEPAX	Güven et al. 2012	E	A	C	Tissue digestion, priming and straining, centrifugation, washing	0.15% NB6 GMP Grade Collagenase	90-120	-	40-400	-	Pellet	-
TGCIS	Doi et al. 2012	E	A	C	Tissue digestion, centrifugation, washing, 700 xg centrifugation	0.075% collagenase	65	D	20-60	-	Pellet	-

AIS Automated Isolation System; CHA-station (CHA-Biotech); CYT Celution System Enzymatic (Cytori); GID SVF2 (GID Europe); LIPOK Lipokit System (Medi-khan); PNC Multi station (PNC); SEPAX Sepax (Biosafe); TGCIS Tissue Genesis Cell Isolation System (Tissue Genesis)

Table 3B. Duration, costs and procedure characteristics of intraoperative concentration procedures focusing on tSVF

Name	Author	Enzymatic/ Non-enzymatic (E/N)	Automatic/ Manual/ Semi (A/M/S)	Open/ closed (O/C)	Isolation details		Time (min)	Disposable (D)/ reusable (R) cost (Dollar)	Volume processed (ml)	Capacity (ml)	End volume (ml)	Maximum volume processed /maximum end volume
FAT	Van Dongen et al. 2016	N	M	O	3000 rpm (radius 9.5 cm) centrifugation, shuffling through a 1.4 mm hole connector, 3000 rpm (radius 9.5 cm) centrifugation	n/a	8-10	R	10	10	0.96 [0.75 - 1.75]	10.4
FAST	Domenis et al. 2015	N	M	-	Filterbag (120 micron filter), 400 xg centrifugation	n/a	-	-	-	-	10	-
FEF	Mashiko et al. 2016	N	M	O	1200 xg centrifugation, shuffling through a connector with three small holes 30 times, 1200 xg centrifugation, fluid of decanting filtration (500-μm pore size) used	n/a	-	-	-	-	-	9.9+/-2.0
LIPOG	Bianchi et al. 2013	N	M	C	Filtering, decantation, stainless steel marbles to mix layers (oil, adipose tissue, blood, saline), washing, decantation, reversing devices, filtering	n/a	20	D	40-130	130	60-100	1.3
NANO	Tonnard et al. 2013	N	M	O	Shufling adipose tissue through a female-to-female luerlok 30 times, filtering	n/a	-	-	-	-	-	-
REF	Mashiko et al. 2016	N	M	O	1200 xg centrifugation, shuffling through a connector with three small holes 30 times, 1200 xg centrifugation, residual tissue of decanting filtration (500-μm pore size) used	n/a	-	-	-	-	-	2.5+/-0.2
SF	Mashiko et al. 2016	N	M	O	1200 xg centrifugation, squeeze using automated slicer, 1200 xg centrifugation	n/a	-	-	-	-	-	2.1+/-0.2
SHUF5	Osinga et al. 2015	N	M	O	Shuffling lipoaspirate through female-to-female luerlok 30 times	n/a	5 sec.	-	10	-	-	-
SHUF30	Osinga et al. 2015	N	M	O	Shuffling lipoaspirate through female-to-female luerlok 30 times	n/a	30 sec.	-	10	-	-	-
STCELL	Milan et al.	N	M	C	1000 xg centrifugation	n/a	-	-	400	500	Pellet	-

FAT Fractionation of Adipose Tissue procedure; FAST Fastem Corios (Corios); FEF Filtrated fluid of emulsified fat; LIPOG Lipogems (Lipogems); NANO Nanofat procedure; REF Residual tissue of emulsified fat; SF Squeezed fat; SHUF5 Shuffling 5 times; SHUF30 Shuffling 30 times

Table 4A: Effect sizes of studies evaluating enzymatic intraoperative isolation procedures regarding cell yield

Study	Enzymatic isolation procedure			Non-intraoperative isolation protocol			Effect size
	N	Cell yield x10^5 cells	SD	N	Cell yield x10^5 cells	SD	
AIS, SundarRaj, 2015	11	1.17	0.5	11	1.15	0.30	0,07
CYT, Domenis, 2015	9	11.7	5.0	16	6.7	3.30	1,52
CYT, Lin, 2008	6	3.7	0.9	3	4.96	0.72	-1,75
LIPOK, Domenis, 2015	9	5.0	3.0	16	6.7	3.30	-0,52
SEPAX, Güven, 2012	6	2.6	1.2	6	1.6	0.90	1,11
TGCIS, Doi, 2012	6	7.0	1.9	6	7.0	2.43	0,00

AIS Automated Isolation System; CYT Celution System Enzymatic (Cytori); LIPOK Lipokit System (Medi-khan); SEPAX Sepax (Biosafe); TGCIS Tissue Genesis Cell Isolation System (Tissue Genesis)

Table 4B. Effect sizes of studies evaluating viable nucleated cells

Study		Procedure			Non-intraoperative isolation protocol			
	N	% viable cells	SD	N	% viable cells	SD	Effect size
Enzymatic								
AIS, SundarRaj, 2015	11	97.5	2.8	11	97.3	1.5	0.13
CYT, Lin, 2008	3	89.2	1.1	3	90.8	1.3	-1.23
TGCIS, Doi, 2012	6	80.7	7.1	6	82.4	7.7	-0.22
Non-enzymatic								
FEF, Mashiko, 2016	10	39.3	9.1	10	93.8	1.2	-45.4
REF, Mashiko, 2016	10	90.6	2.8	10	93.8	1.2	-2.67
SF, Mashiko, 2016	10	89.9	4.6	10	93.8	1.2	-3.25
STCELL, Millan, 2014 [a]	3	87.7	8.9	3	74.5	20.1	0.66

[a] No exact data described in text, data extracted from figures by authors JAD and AJT. AIS Automated Isolation System; CYT Celution System Enzymatic (Cytori); FEF Filtrated fluid of emulsified fat; REF Residual tissue of emulsified fat; SF Squeezed fat; STCELL StromaCell; TGCIS Tissue Genesis Cell Isolation System (Tissue Genesis)

Studies	Viability	Flow cytometry of SVF	Flow cytometry of cultured ASCs									CFU-F	Functional assasys			Total Score
			CD 13	CD 29	CD 31	CD 44	CD 45	CD 73	CD 90	CD1 05	CD2 35a		Adipog enic	Osteog enic	Chondro genic	
Aronowitz et al. 2013	1	1										1				3.00
Aronowitz et al. 2016	1	1										1				3.00
Bianchi et al. 2013	1	1										0	1/3	1/3	1/3	3.00
Doi et al. 2012	1	1										0				2.00
Domenis et al. 2015	0	1	1/9	1/9		1/9	1/9	1/9	1/9	1/9		1				2.78
Van Dongen et al. 2016	1	0		1/9	1/9	1/9	1/9		1/9	1/9		1	1/3	1/3		3.33
Güven et al. 2012	1	1		1/9		1/9		1/9	1/9	1/9		1	1/3	1/3	1/3	4.56
Lin et al. 2008	1	1										1	1/3	1/3		3.67
Mashiko et al. 2016	1	1										0				2.00
Millan et al.	1	0				1/9		1/9	1/9	1/9		0				1.44
Osinga et al. 2015	1	0										1	1/3	1/3	1/3	3.00
SundarRaj et al. 2015	1	1										1				3.00
Tonnard et al. 2013	0	1										0	1/3			1.33